Differential determination of phospholipase A_2 and PAF-acetylhydrolase in biological fluids using fluorescent substrates

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Abstract **The purpose of the present study was the development and evaluation of a fluorimetric method for the screening and differential determination of phospholipase A_2 and PAF-acetylhydrolase in bronchoalveolar lavage (BAL) fluid and serum. Phospholipase A_2 was determined using C_{12}-NBD-PC in the presence of Ca^{2+}, from the slope of the fluorescence enhancement due to the formation of C_{12}-NBD-fatty acid. PAF-acetylhydrolase was determined using C_6-NBD-PC, from the slope of the curve due to C_6-NBD-fatty acid formation in the absence of Ca^{2+}. The results were confirmed after TLC analysis. The method's selectivity was evaluated by comparing to radiometric measurements. Light scattering did not interfere and inner filter effects was not observed under our experimental conditions. The effects of pH, temperature, and Ca^{2+} were investigated. Protein caused an increase in the background fluorescence of both NBD-PCs. The standard curves of both NBD-fatty acids exhibited the same slope. Linearity extended at least up to 4.5 nmoles per ml of reaction mixture at the normal pH 7.4. The fluorescence of the NBD-fatty acids remained stable for increasing concentrations of BAL fluid and serum and for BSA up to 100 μg/ml of reaction mixture. Porcine pancreatic PLase A_2 showed preference for C_{12}-NBD-PC in the presence of Ca^{2+}, while without Ca^{2+}, serum PAF-AcH hydrolyzed only C_6-NBD-PC. The method is highly sensitivite, accurate, and reproducible and can be applied for the differential determination of phospholipase A_2 and PAF-acetylhydrolase activities in BAL fluid and serum.**—Kitsiouli, E. I., G. Nakos, and M. E. Lekka. **Differential determination of phospholipase A_2 and PAF-acetylhydrolase in biological fluids using fluorescent substrates.** ***J. Lipid Res.*** **1999.** 40: **2346–2356.**

Supplementary key words phospholipase A_2 • PAF-acetylhydrolase • bronchoalveolar lavage • NBD-lipids • fluorescence • phosphatidylcholines

Phospholipases A_2 (PLases A_2) are the enzymes that stereospecifically catalyze the hydrolysis of *sn*-2 acyl ester bonds from glycerophospholipids, producing free fatty acids and lyso-glycerophospholipids. They play a significant role in lipid metabolism and membrane repair, as well as in the formation of potent inflammatory lipid mediators, such as the arachidonic acid metabolites and platelet-activating factor (PAF).

They are found in important quantities in mammalian pancreas and in venom glands, in bacterial secretions and digestive fluids of higher organisms. So far, PLases A_2 are classified into ten groups (1, 2), based on their sequence homology. Groups, I, II, and III contain exclusively secreted, Ca^{2+}-dependent forms of the enzyme. Group IV comprises cytosolic PLases A_2 with preference for arachidonic acid, but they additionally manifest PLase A_1 and transacylase activity. Group VI Ca^{2+}-independent PLase A_2 displays interfacial activation towards long chain phospholipids, exhibiting also lysophospholipase and transacylase activity (3). Two other groups of PLases A_2 that are Ca^{2+}-independent show preference for short *sn*-2 acyl chains, particularly for acetyl residues, and they are called PAF-acetylhydrolases (PAF-AcH) because of their capability to degrade PAF. These groups include a secreted form present in plasma (4, 5), which displays anti-inflammatory properties, as well as intracellular forms (6, 7). PAF is a potent phospholipid mediator in inflammatory and allergic reactions, and is implicated in acute as well as in long-

Abbreviations: ARDS, adult respiratory distress syndrome; BSA, bovine serum albumin; HSA, human serum albumin; C_6-NBD-PC, 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] hexanoyl]-*sn*-glycero-3-phosphocholine; C_{12}-NBD-PC, 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine; C_6-NBD-FA, 6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoic acid; C_{12}-NBD-FA, 12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoic acid; DPPC, dipalmitoyl-phosphatidylcholine; BAL(F), bronchoalveolar lavage (fluid); PLase A_2, phospholipase A_2; AcH, acetylhydrolase; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; [^{3}H]PAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol-3-phosphorcholine-[acetyl-1-^{3}H]; TCA, trichloroacetic acid; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediamino-tetraacetic acid; PBS, phosphate buffer saline; CMC, critical micellar concentration; C-M-W, chloroform–methanol–water; FA, fatty acid; AcA, acetic acid; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; TLC, thin-layer chromatography; A_{475}, absorbance at 475 nm.

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term pulmonary effects. PAF causes bronchoconstriction, increased vascular permeability, and recruitment of inflammatory cells, especially eosinophils, neutrophils, and platelets in the lungs (8).

Bronchoalveolar lavage (BAL) fluid represents alveolar fluid in dilution, containing lung surfactant, cells that are inherent to the alveoli, albumin, and other proteins. Surfactant, a material with good surface properties that lines lungs, lowers surface tension and thus protects alveoli from collapsing, especially at the end of expiration. In addition, it participates in host-defense mechanisms. It is composed mainly of phospholipids (90%), especially dipalmitoyl-phosphatidylcholine (DPPC) (70%) and phosphatidylglycerol (10%), minor quantities of PS, PI, PE, specific surfactant, and proteins. It is biosynthesized by alveolar type II cells and excreted in the alveolar space in the forms of lamellar bodies and tubular myeline. When these structures reach the alveolar water/air interface, the bilayers unfold and surfactant monolayer spreads on the surface (9). During a lung insult, the permeability of capillary-alveolar membrane may be disturbed and consequently the protein concentration in the alveoli may be dramatically altered. Recruitment of inflammatory cells may also occur and inflammatory mediators, like platelet-activating factor (10), cytokines, enzymes as well as autoantibodies (11) may emerge.

PLases A_2 play a significant role in the pathogenesis or in the perpetuation of lung inflammation and this directly affects surfactant balance (12). A secretory PLase A_2 was isolated and characterized from BAL fluid of human healthy subjects (13). Elevated PLase A_2 levels were detected in plasma and BAL fluid from humans with septic shock (14, 15). Elevated levels were also found in experimental models of allergic asthma induced with ovalbumin (16). PAF-AcH was detected in BAL fluid from human subjects with hydrostatic pulmonary edema (17), ARDS (10), asthma, lung fibrosis (18), and pulmonary embolism (19).

PLases A_2 are soluble in aqueous environments, but their activity is greatly enhanced on lipid interfaces. Therefore, the substrates are applied in concentrations above the critical micellar concentrations (CMC). Radiometric methods are widely used for the determination of the various forms of PLases A_2 and PAF-AcH as they provide high sensitivity and selectivity, but they are laborious, often require chromatographic purification of the products, have radiochemical hazards, and are expensive. Fluorimetric assays are also sensitive (20), although they can be influenced by physicochemical parameters that affect fluorescence as a physical magnitude, such as temperature, pH, micellar composition of the substrate, presence of protein, etc. This could complicate the analysis of BAL fluid samples, in which phospholipids and proteins, the main constituents, are organized in micelles or vesicles, structures that potentially facilitate the hydrolysis by phospholipases. Wittenauer et al. (21) developed a method for the determination of porcine pancreatic PLase A_2 and purified lipoprotein lipase from skimmed milk, using C_6-NBD-PC as substrate. Moreau (22) studied some properties of the fluorescent C_6-NBD-lipids as substrates for PLase A_2, PLase C, PLase D, and lipases under different experimental conditions, while Blanchard, Harris, and Parks (23) used NBD-PE and PC species for the investigation of human synovial PLase A_2 characteristics. The capability of PAF-AcH to cleave C_6-NBD-PC was found during the investigation of the enzymic activities that mediate PC hydrolysis during LDL oxidation (24).

The purpose of the present study was the development and application of a fluorimetric method for the screening and the differential determination of PLase A_2 and PAF-AcH in BAL fluid and serum from patients with acute lung injury. In this direction, the discrimination between PLase A_2 and PAF-AcH activity was assessed using the fluorescent C_6-NBD-PC and C_{12}-NBD-PC as substrates. The inner filter effect and light scattering interference as well as the effect of pH, temperature, Ca^{2+}, and protein concentration in the reaction mixture were investigated. This fluorimetric method was evaluated by comparing to a radiometric method.

MATERIALS

BSA, HSA, EDTA, Tris-HCl, standard lipids, and inorganic compounds were purchased from Sigma Chemical Co. (St. Louis, MO). Solvents were from Merck. 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine was from Bachem (Switzerland) and 1-*O*-hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (7.1 Ci/mmol) was from New England Nuclear (Boston, MA), and C_6-NBD-PC, C_6-NBD-FA, C_{12}-NBD-PC, and C_{12}-NBD-FA were from Avanti Polar Lipids (Pelham, AL).

METHODS

Total protein was determined by the method of Lowry et al. (25) using BSA as a reference compound. Total lipids were extracted according to Bligh and Dyer (26). Phospholipids were measured according to Bartlett (27). Polar and neutral lipids were analyzed with TLC on silica gel 60 precoated plates (1.05721, Merck, Germany), using chloroform–methanol–water 65:35:7 (v/v) and chloroform–methanol–acetic acid 95:5:1 (v/v) as solvent systems, respectively. The same solvent systems were used for the verification of the purity of the authentic NBD-lipids. Excitation spectra were obtained at 60 nm/min scanning speed and a slit-width of 10 nm by adjusting the emission wavelength at 535 nm. Emission spectra were obtained by adjusting the excitation wavelength at 475 nm. Measurements of the fluorescent samples were obtained under continuous excitation. The CMC of C_6-NBD-PC was determined by plotting the fluorescence versus lipid concentration and extrapolating the lines corresponding to low and high concentrations to the intersection point (28). BAL fluid was obtained by fibreoptic bronchoscopy from intubated patients with acute lung injury (17).

Phospholipase A_2 for long *sn*-2 acyl chains (PLase A_2)

Substrate concentrations were adjusted above the critical micellar ones. Vesicles of the substrates were formed with the addition of 10 μl ethanol solution of the relative substrate in the aqueous medium (29). In routine assays, commercial porcine PLase A_2 (1 μg, 0.08 IU) serving as a reference enzyme, 60 μg BAL protein, or serum diluted 1:50 were used. PLase A_2 was determined with two different methods.

Fluorimetric method. A modification of the method of Wittenauer et al. (21) was applied. PLase A_2 hydrolysis of the fluorescent phospholipids resulted in approximately a 50-fold enhancement in fluorescence intensity, due to the liberation of NBD-hexanoic and NBD-dodecanoic acids, respectively, without shift in the emission maximum. In the present work, C_6-NBD-PC or C_{12}-NBD-PC were used as substrates. Standard incubation mixture, 1 ml final volume (Hellma cuvette 104F-OS, 10 mm light path, Windaus Labortechnik GMBH & Co., Mullheim/Baden), 10 mm Tris-HCl buffer, pH 7.4, with 2 mm Ca^{2+} and 5 μm NBD-PC was used. For experiments performed in the absence of Ca^{2+}, a final concentration of 10 mm EDTA was applied. The reaction started with the addition of 100 μl from the source of the enzyme, at 25°C temperature, and was continuously monitored with a Perkin-Elmer LS-3 spectrofluorimeter, equipped with xenon lamp and a RCA 931 photomultiplier. Excitation and emission wavelengths were adjusted to 475 nm and 535 nm, respectively, as resulted from the equivalent spectra. Purity of the authentic substrates was routinely checked with TLC analysis. The absorbance of all the reaction mixtures at 475 nm was measured. The interference of inner filter effect or light scattering was checked experimentally with the application of internal and external standards of the NBD-species.

Exitation and emission spectra of authentic substrates, products, and NBD-lipid mixtures in the presence of BAL fluid, serum, or BSA were obtained at pH 7.4.

In certain experiments the fluorescence of the reaction products was measured after TLC separation. The reaction was stopped with 50 μl of 2 N HCl and the reaction mixture was extracted according to Bligh and Dyer (26). The lipids were spotted on a TLC plate and developed with C–M–W 65:35:7 (v/v). The compounds migrating to the R_f of the relevant NBD-FAs were extracted from the plates and measured fluorimetricaly. Whenever needed, a standard amount of C_6-NBD-FA or C_{12}-NBD-FA was added at the end of the monitoring of the reaction, as an internal standard.

The role of pH, temperature, chemical constituents of the reaction mixture, Ca^{2+}, as well as protein concentration was investigated.

Radiometric method. The standard assay conditions included 10 mm Tris-HCl buffer, pH 7.4, with 2 mm $CaCl_2$, and 5 μm dipalmitoyl-PC as substrate, containing 120,000 cpm (approximately 0.07 μCi in dipalmitoyl-[1-^{14}C]phosphatidylcholine). For experiments performed in the absence of Ca^{2+}, a final concentration of 10 mm EDTA was applied. The reaction was started by the addition of the source of the enzyme. Final volume was adjusted to 1 ml and the reaction was stopped after 5 min for commercial porcine PLase A_2, at room temperature, with 50 μl 2 N HCl followed by the addition of 2.2 ml of chloroform–methanol 1:1 (v/v). After phase separation, the chloroform-rich phase was collected and the lipids were analyzed by TLC, using chloroform–methanol–water 65:35:7 (v/v) as solvent system. The lipids corresponding to the R_f of dipalmitoyl-PC, lyso-PC, and free fatty acids were scraped off the plates and the radioactivity was measured with a TriCarb, Hewlett-Packard liquid scintillation counter. Positive as well as negative controls were used throughout the study. Purity of the substrate was routinely checked with TLC. The background radiolysis of the radiolabeled compounds was found to be around 1%.

PAF-acetylhydrolase activity (PAF-AcH)

Fluorimetric method. C_6-NBD-PC and C_{12}-NBD-PC were evaluated as substrates for PAF-AcH activity, both in the presence and in the absence of Ca^{2+}, according to the protocol described above.

Radiometric method. The enzyme was determined according to Tselepis, Lekka, and Tsoukatos (6), after TCA precipitation. Briefly, 100 μl of serum diluted 1:50 was incubated with an equal volume of 20 μm PAF solution in 1 mg/ml HSA-PBS, pH 7.4, 0.1 μCi [^{3}H]PAF per sample. The reaction was stopped after the indicated time intervals, usually 5 min, with ice-cold TCA. The radioactivity of the acetyl group was measured at the 12,000 *g* supernatant, after centrifugation for 2 min. The unreacted substrate sedimented with denatured HSA. Human serum PAF-AcH was used as reference with an enzymic activity of 30 nmoles PAF per ml serum per min.

RESULTS

Lipid purity and lipid extraction

TLC analysis confirmed the purity of the authentic NBD-lipids. In C–M–W 65:35:7 (v/v) C_{12}-NBD-PC migrated to a R_f value of 0.40, corresponding to authentic PC, while C_6-NBD-PC migrated to R_f 0.35. C_6-NBD-FA migrated to R_f 0.75, while C_{12}-NBD-FA migrated to R_f 0.92, like authentic palmitic acid. In C–M–AcA 95:5:1 (v/v), the fatty acids were very well separated: C_6-NBD-FA migrated to R_f 0.11, C_{12}-NBD-FA to R_f 0.33, and palmitic acid to R_f 0.57. Acidic conditions during the extraction of the NBD-FAs were necessary because HCl facilitated the migration of C_6-NBD-FA into the chloroform phase. Otherwise, in neutral pH values, it was distributed quantitatively to the aqueous phase. This was not observed with C_{12}-NBD-FA, which migrated to the chloroform phase in both conditions.

Spectra

The following excitation and emission spectra were obtained under the experimental conditions described in Methods, for the C_6- as well as the C_{12}-NBD fluorescent lipids: *a*) 5 nmoles C_6-(or C_{12})-NBD-PC; *b*) 0.5 nmoles C_6-(or C_{12})-NBD-FA; *c*) 5 nmoles C_6-(or C_{12})-NBD-PC + 0.5 nmoles C_6-(or C_{12})-NBD-FA; *d*) 0.5 nmoles C_6-(or C_{12})-NBD-FA + BAL fluid (500 μg protein/ml of incubation buffer); *e*) 0.5 nmoles C_6-(or C_{12})-NBD-FA + 100 μl serum (diluted 1:50); *f*) 0.5 nmoles C_6-(or C_{12})-NBD-FA + BSA (30–200 μg/ml of incubation buffer); *g*) 5 nmoles C_6-(or C_{12})-NBD-PC + BAL fluid (500 μg protein/ml of incubation buffer); *h*) 5 nmoles C_6-(or C_{12})-NBD-PC + 100 μl serum (diluted 1:50); and *i*) 5 nmoles C_6-(or C_{12})-NBD-PC + BSA (100 μg/ml of incubation buffer). Emission spectra were also taken for a mixture containing the four fluorescent substances, BAL fluid, and serum (diluted 1:50) using the same quantities as above. In the presence of BAL fluid (up to 500 μg protein) and serum there was not any significant difference at the excitation and emission maxima of the incubation mixtures. In the presence of BSA concentrations higher than 100 μg per ml of incubation mixture, the emission maximum of C_{12}-NBD-FA shifted by 15 nm to a lower wavelength. On the other hand, the emission maxima of C_6-NBD-FA, C_6-NBD-PC, and C_{12}-NBD-PC did not shift. The presence of Ca^{2+} or EDTA did not alter the above results.

Properties of the fluorescence of the authentic NBD-lipids

The effect of the following parameters on the fluorescence intensity of the authentic NBD-lipids was evaluated.

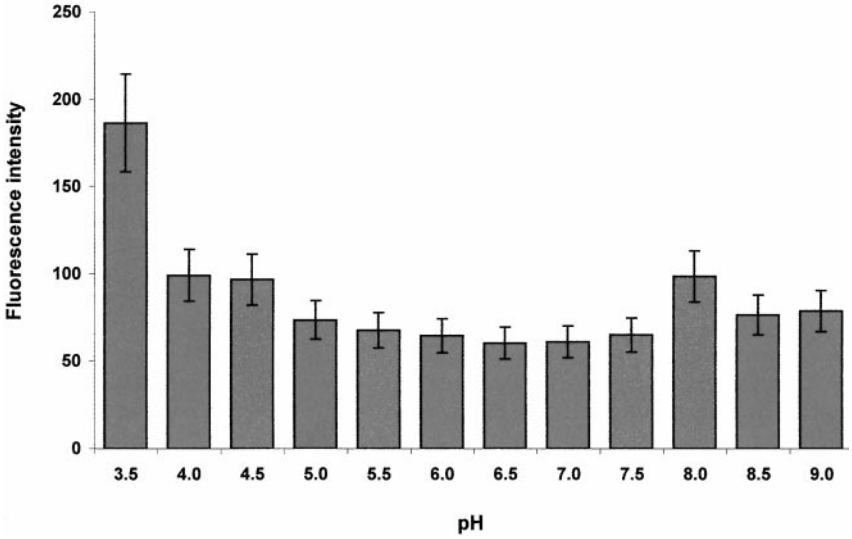

Fig. 1. Effect of pH on the fluorescence of C_6-NBD-PC in the presence of BAL at 25°C. The 1 ml final volume of buffer solution contained 5 μm C_6-NBD-PC and 40 μg BAL protein. Acetate buffer solution (0.1 m) was used for pH values ranging from 3.5–5.5; 0.1 m phosphate buffer solution was used for pH 5.5–7.0 and 10 mm Tris-HCl buffer solution was used for pH 7.0–9.0, respectively. Values were obtained before the reaction could be monitored and represent the mean of three measurements. NBD-PC fluorescence was subtracted from the measurements.

Components of the reaction mixture. Calcium ions, KCl, or EDTA did not influence the fluorescence of given concentrations of C_6-NBD-PC or C_{12}-NBD-PC.

Temperature. A linear decrease in fluorescence intensity for both NBD-FAs was obtained with the increase of temperature. In particular, at 43°C the fluorescence was 34% lower than at 20°C. Regarding the NBD-PCs, the corresponding decrease was 14%.

Sonication. After sonication for 30 sec in a bath sonicator (Fritsch Laborette, type 17.202, Germany), a 25% increase in the fluorescence level of C_6-NBD-PC was observed, while for C_{12}-NBD-PC the increase was not significant. The fluorescence of the relevant NBD-FAs was not affected.

pH. Most of the experiments were performed at the normal pH value, 7.4. When different pH values were applied, the fluorescence intensity of NBD-PCs was not altered. In the presence of BAL protein, however, at pH 3.5 and before the initiation of the reaction, a remarkable increase in the fluorescence of the reaction mixture containing the substrates and BAL fluid was observed. This increase did not correspond to product formation, as the TLC analysis of the reaction mixture revealed. An increase in the background fluorescence, ranging from 15–25%, was also observed at pH values 4.0, 4.5, and 8.0 in comparison to pH 7.4. Between 5.0 and 7.5 the fluorescence was stable (**Fig. 1**). Regarding the NBD-FAs, equal quantities of both gave the

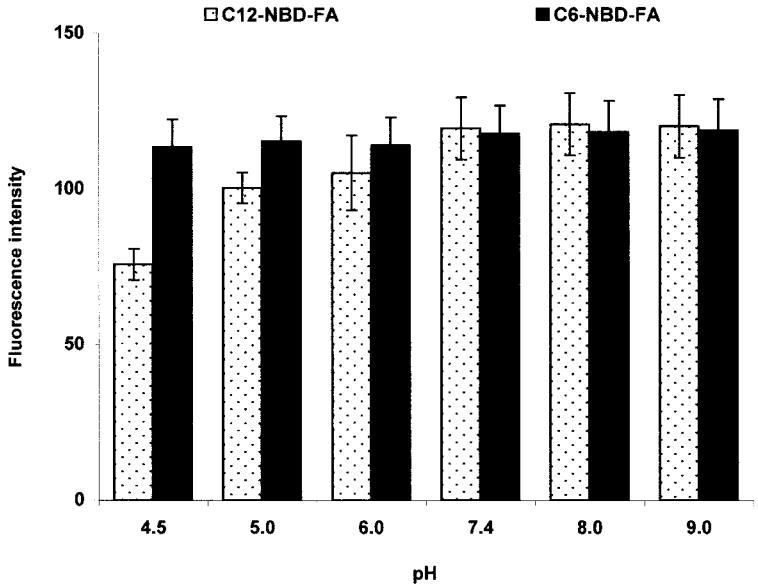

Fig. 2. Effect of pH on the fluorescence of NBD-FAs at 25°C. The incubation mixture contained 5 μm NBD-PC. Acetate buffer solution (0.1 m) was used for pH values ranging from 3.5–5.5; 0.1 m phosphate buffer solution was used for pH 5.5–7.0 and 10 mm Tris-HCl buffer solution was used for pH 7.0–9.0, respectively. Values represent the mean of three measurements. NBD-PC fluorescence was subtracted from the measurements.

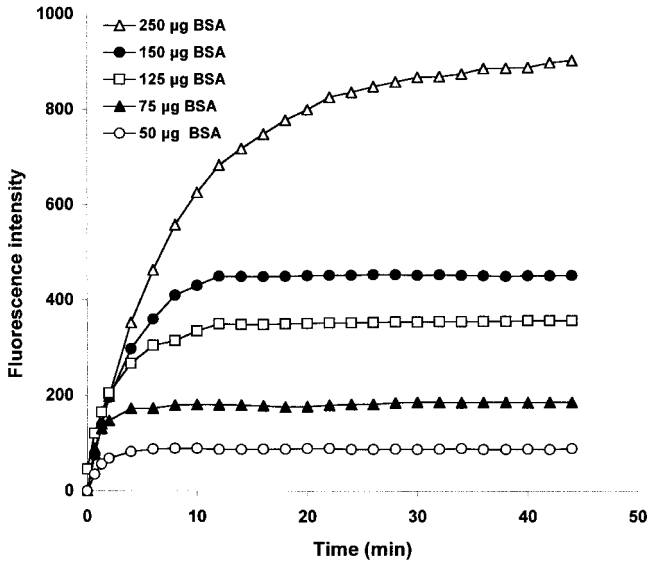

Fig. 3. Effect of BSA concentration on the fluorescence intensity of C_{12}-NBD-PC at 25°C. The 10 mm Tris-HCl buffer solution, pH 7.4, contained 2 mm Ca^{2+} or 10 mm EDTA, 5 μm C_{12}-NBD-PC, and increasing BSA concentrations. The fluorescence enhancement of the incubation mixture was monitored directly.

same fluorescence intensity in neutral as well as in alkaline pH regions. The fluorescence of C_{12}-NBD-FA was reduced in the acidic region, parallel to the acidity of the incubation mixture (**Fig. 2**), unlike C_6-NBD-FA which was not affected.

Effect of BSA on the fluorescence of authentic NBD-PCs

Serum and BAL fluid protein consist of approximately 50% and 20–30% albumin, respectively. Considering the fact that albumin functions as a lipid carrier molecule, its effect on the fluorescence of the NBD-PCs and the NBD-FAs was investigated in a series of experiments.

As soon as BSA was added to a buffer solution containing authentic C_6- or C_{12}-NBD-PC, an abrupt increase in the fluorescence level occurred. Subsequently, after a few minutes, the slope of the curve changed to reach a plateau (**Fig. 3**). The time interval in which the initial fluorescence increase was accomplished differed according to the protein concentration. In addition, the level of the initial fluorescence increase, for a given NBD-PC concentration, was proportional to BSA concentration. The curve of C_6-NBD-PC fluorescence versus BSA concentration was linear from 20 to 80 μg BSA/ml buffer solution, while for C_{12}-NBD-PC, from 50 to 150 μg BSA/ml buffer (**Fig. 4**). The slopes of the two curves differed significantly. In addition, the absorbance of 5 μm of C_6- or C_{12}-NBD-PC in the buffer solution at 475 nm was around 0.06 and it did not increase after the addition of BSA. Therefore, the decline from the linear response above 80 or 150 μg BSA/ml incubation buffer was not due to inner filter effect.

Effect of BAL fluid, serum, and BSA on the fluorescence of authentic NBD-FAs

In a buffer solution containing 5 μm C_6- or C_{12}-NBD-PC and increasing concentrations of BAL fluid, serum, or BSA, 0.5 nmole of each NBD-FA was repeatedly added. The fluorescence enhancement after each addition was the same when equal amounts of the NBD-FAs were added in the buffer solution in the absence of BAL fluid or serum. Linearity of the fluorescence extended up to at least 4.5 nmoles of each NBD-FA per ml of reaction mixture (**Fig. 5**).

However, the fluorescence intensity of C_{12}-NBD-FA in the presence of high BSA concentrations (>100 μg protein per ml of reaction mixture) and at its emission maximum, which shifted at 520 nm, was increased approximately by 80%. Of note is that when the fluorescence of the above incubation mixture was measured at 535 nm, the values obtained were similar to those of C_{12}-NBD-PC at 535 nm, without the presence of BSA.

The addition of BAL fluid or serum diluted 1:50 or BSA did not cause an increase in the absorbance of the buffer solution containing 5 μm C_6- or C_{12}-NBD-PC at the wavelength of excitation (475 nm). The absorbance at 475 nm of 0.5 nmoles C_6- or C_{12}-NBD-FA in the buffer solution was 0.012. Repeated additions of 0.5 nmoles of C_6- or C_{12}-

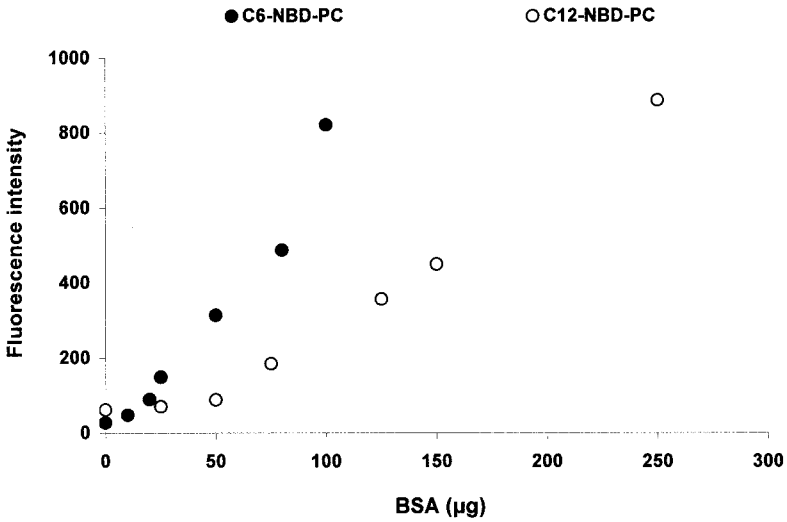

Fig. 4. Effect of BSA concentration on the fluorescence intensity of C_{12}-NBD-PC and C_6-NBD-PC at 25°C. Each point represents a fluorescence value after the curve of the fluorescence versus protein concentration reached a plateau with continuous monitoring. The incubation mixture contained 10 mm Tris-HCl buffer solution, pH 7.4, 2 mm Ca^{2+} or 10 mm EDTA, 5 μm NBD-PC and increasing BSA concentrations.

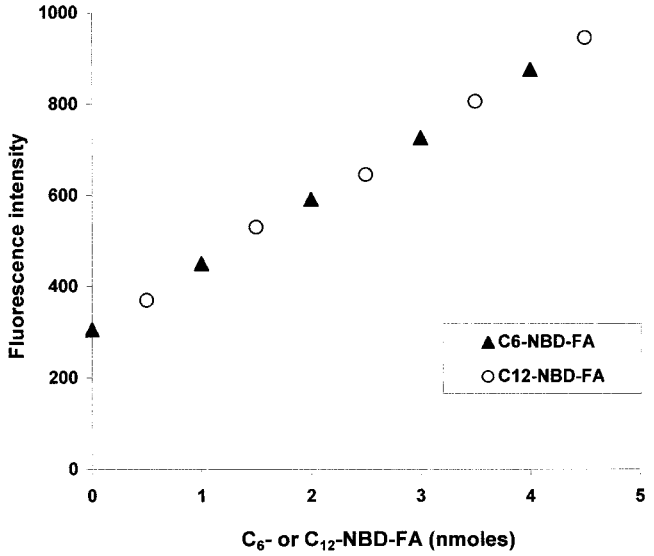

Fig. 5. Standard curves of NBD-FAs in the presence of BAL fluid and both substrates at 25°C. The incubation mixture (1 ml) contained 10 mm Tris-HCl buffer solution, pH 7.4, 2 mm Ca^{2+} or 10 mm EDTA, 5 μm C_6-NBD-PC, 5 μm C_{12}-NBD-PC, and 100 μl of BAL fluid (500 μg protein). After the fluorescence increase of the substrates due to the presence of BAL protein had been stabilized (zero time), 5 μl of 0.1 mm C_6-NBD-FA or C_{12}-NBD-FA ethanol solution was added in the mixture alternately. Fluorescence values represent the mean of three measurements.

NBD-FA in the mixture containing BSA, BAL, or serum caused a concentration-dependent increase of the A_{475} by the same amount.

The linear response of the fluorescence extended even up to 4.5 nmoles of NBD-FA, although the A_{475} at that concentration was 0.200. This corresponded to the near total hydrolysis of the substrate (5 nmoles) and it was out of the detection range of our method.

Effect of BAL fluid on the fluorescence of authentic NBD-PCs

When BAL fluid instead of BSA was included in the incubation mixture containing the substrate, an initial increase in the fluorescence intensity was observed, before the direct monitoring of the reaction was feasible. Therefore, measurements of PLase A_2 activity were obtained after the time in which the change of the slope of the curve was stabilized.

The intersection point of the lines corresponding to the initial increase in fluorescence (due to the presence of proteins) and the linear component representing the enzymic reaction, increased linearly with BAL fluid protein concentration, up to 500 μg protein per ml of reaction mixture (**Fig. 6**).

The absorbance of the reaction mixture at the wavelength of excitation (475 nm) did not change significantly during the first minutes of the addition of BAL fluid when the abrupt increase in fluorescence intensity was observed. BAL fluid alone in the buffer solution, without the presence of NBD-lipids, neither emitted any signal at 535 nm nor absorbed at 475 nm.

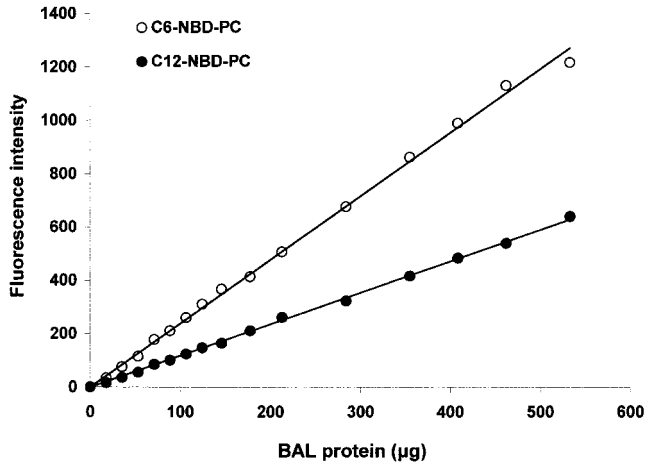

Fig. 6. Effect of BAL protein concentration on the fluorescence intensity of NBD-PCs at 25°C. The incubation mixture (1 ml) contained 10 mm Tris-HCl buffer solution, pH 7.4, 2 mm Ca^{2+} or 10 mm EDTA, 5 μm NBD-PC, and increasing BAL fluid protein concentration. Each point represents a fluorescence value after the curve of the continuous fluorescence monitoring had reached a plateau and before the reaction could be monitored.

After the first increase, the curve changed to a linear component with a slope corresponding to product formation. This was confirmed by a series of experiments in which the products of the reaction were isolated after acidic lipid extraction and TLC purification, at different time intervals. The slope of the curve obtained after continuous fluorescence monitoring of the reaction mixture was identical to that obtained after product purification (**Fig. 7**). While the product was liberated, the absorbance of the reaction mixture at 475 nm increased slightly but

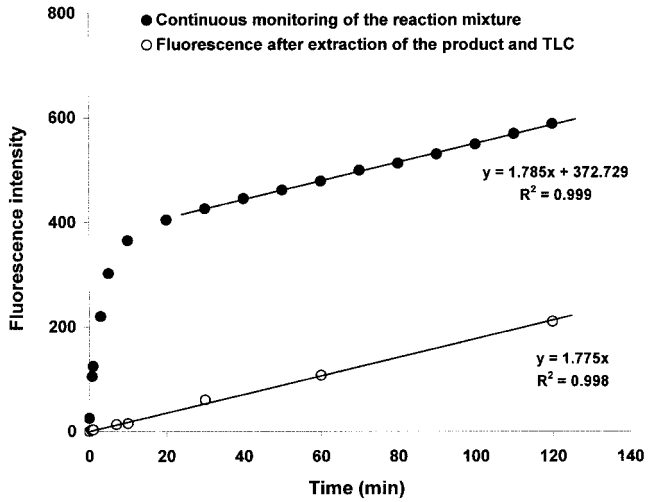

Fig. 7. BAL-PLase A_2 activity at 25°C. Direct monitoring of the fluorescence (at 535 nm) of the reaction mixture with continuous excitation at 475 nm (●) and measurement of the fluorescence of the isolated product after TLC separation (○), at different time intervals. The incubation mixture (1 ml) contained 10 mm Tris-HCl buffer solution, pH 7.4, 2 mm Ca^{2+}, 5 μm C_6-NBD-PC, and 100 μl of BAL fluid as a source of the enzyme.

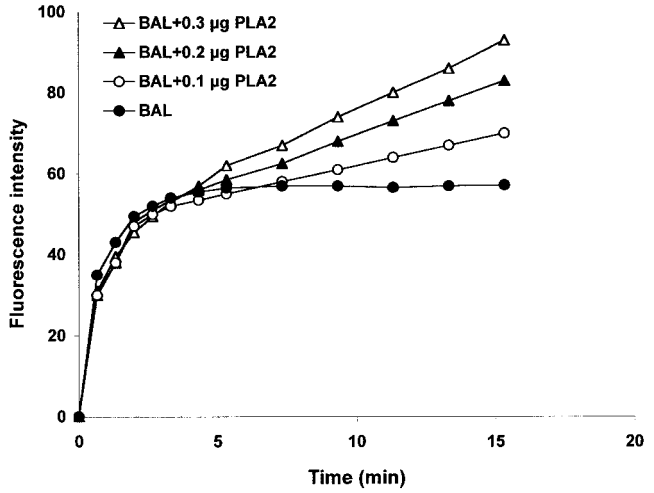

Fig. 8. Porcine pancreatic PLase A_2 activity in the presence of BAL fluid at 25°C. The incubation mixture (1 ml) contained 10 mm Tris-HCl buffer solution, pH 7.4, 2 mm Ca^{2+}, 5 μm C_6-NBD-PC, BAL fluid, and increasing porcine pancreatic PLase A_2 concentrations. BAL fluid was selected so that it did not present any PLase A_2 activity. NBD-PC fluorescence was subtracted from the measurements.

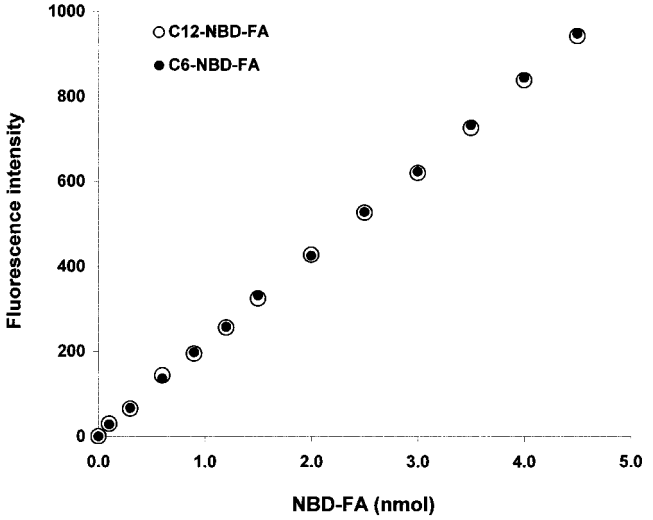

Fig. 9. Standard curves of NBD-FAs at 25°C. The incubation mixture (1 ml) contained 10 mm Tris-HCl buffer solution, pH 7.4, 2 mm Ca^{2+} or 10 mm EDTA, 5 μm C_6-NBD-PC (or C_{12}-NBD-PC) as well as increasing C_6-NBD-FA (or C_{12}-NBD-FA) amounts, respectively. Values represent the mean of three measurements. NBD-PCs fluorescence was subtracted from the measurements.

never exceeded 0.100 throughout this experiment. When BAL fluid sample free of PLase A_2 activities was included in the incubation buffer, there was no false positive reaction. In the presence of increasing concentrations of porcine pancreatic PLase A_2, the slopes of the curves increased proportionally (**Fig. 8**).

Standard curves.

C_6- and C_{12}-NBD-FAs. Standard curves of authentic C_6-NBD-FA and C_{12}-NBD-FA versus fluorescence, at pH value 7.4, were prepared. As shown in **Fig. 9**, the fluorescence of both NBD-FAs suspended in buffer solution increased linearly within a wide range of concentrations, at least up to 4.5 nmoles NBD-FA per ml. The two curves coincided. The minimum quantity of NBD-FAs that could be measured by the fluorimeter was 100 pmoles for each acid. The A_{475} of 0.5 μmoles C_6-NBD-FA or C_{12}-NBD-FA per ml of buffer solution was 0.012.

Authentic C_6- and C_{12}-NBD-PC. For C_6-NBD-PC, the curve of the fluorescence intensity versus substrate concentration was linear up to 0.1 μm. Above 0.5 μm the slope changed, indicating a modification in the physicochemical status of the solution. The intercept of the two curves corresponding to the CMC of C_6-NBD-PC was found to correspond to 0.2 μm. For C_{12}-NBD-PC, the curve was linear right from the beginning, indicating that the CMC was lower than the threshold of the fluorimetric method applied. The slopes of the two curves differed significantly. In routine experiments, the initial concentration of C_6- or C_{12}-NBD-PC was 5 μm, which was far above the CMC (**Fig. 10**). The A_{475} of 0.5 μmoles C_6-NBD-PC and C_{12}-NBD-PC per ml of buffer solution was 0.061 and 0.064, respectively.

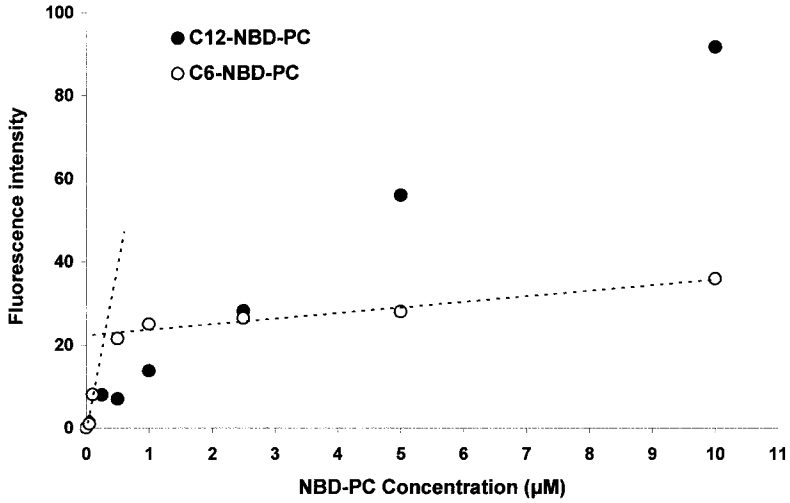

Fig. 10. Critical micellar concentration (CMC) of C_6-NBD-PC at 25°C. The incubation mixture (1 ml) contained 10 mm Tris-HCl buffer solution, pH 7.4, 2 mm Ca^{2+}, and increasing C_6-NBD-PC or C_{12}-NBD-PC concentrations, respectively. Values represent the mean of three measurements.

TABLE 1. Substrate selectivity of porcine pancreatic PLase A_2 and serum PAF-AcH

	Enzymic Activity			
	Serum PAF-AcH		Pancreatic PLase A_2	
Substrate	10 mm EDTA	2 mm Ca^{2+}	10 mm EDTA	2 mm Ca^{2+}
	nmol/ml serum/min		*nmol/mg protein/min*	
[^{14}C]dipalmitoyl-PC	0	0	0	504 ± 84
C_{12}-NBD-PC	0	0	0	387 ± 77
C_6-NBD-PC	15.3 ± 2.5	13.5 ± 2.0	0	111 ± 12
[^{3}H]PAF	33.7 ± 3.2	32.5 ± 3.0	0	2.0 ± 0.1

Serum PAF-AcH was tested with and without Ca^{2+} in the incubation buffer, while porcine pancreatic PLase A_2 in the presence of 2 mm Ca^{2+}, at room temperature. Values represent mean ± SD of four determinations.

Control experiments with commercial porcine pancreatic PLase A_2 and serum PAF-AcH

Porcine pancreatic PLase A_2 and serum PAF-AcH were used as reference enzymes. When porcine pancreatic PLase A_2 or serum diluted 1:50 was added in the buffer solution there was no detectable signal at 535 nm.

Substrate specificity and the role of Ca^{2+}

The specific activities of authentic PLase A_2 and serum PAF-AcH were determined using the NBD-fluorescent and radiolabeled substrates, in the presence or in the absence of Ca^{2+}. Depletion of Ca^{2+} was achieved with 10 mm EDTA as a final concentration in the reaction mixture. The presence of EDTA was essential, as even the constitutive levels of Ca^{+}, when neither Ca^{2+} nor EDTA were added, allowed certain levels of the enzymic activity. Porcine pancreatic PLase A_2 in the absence of Ca^{2+} was totally inactive towards all the substrates. In the presence of Ca^{2+}, the activity increased in positive relation to the chain length, as shown in **Table 1**. It is noteworthy that PAF was poorly recognized as a substrate for PLase A_2. On the other hand, serum PAF-AcH was not capable of hydrolyzing C_{12}-NBD-PC and Ca^{2+} did not reverse this behavior even after 4 h of continuous monitoring of the reaction. However, serum PAF-AcH recognized C_6-NBD-PC in both the presence and absence of Ca^{2+}.

Simultaneous determination of serum PAF-AcH and porcine pancreatic PLase A_2

In order to determine both PLase A_2 and PAF-AcH activity in the same sample, experiments were conducted in an incubation buffer containing 10 mm Tris-HCl, 2 mm Ca^{2+}, pH 7.4, 2 μg porcine pancreatic PLase A_2, and serum, as a source of PAF-AcH, diluted 1:50. The reaction started with the addition of 5 μm C_{12}-NBD-PC and was monitored directly. The slope of the curve represented the activity of PLase A_2 cleaving long chains at the *sn*-2 position. After depletion of Ca^{2+} with 30 mm EDTA final concentration, at pH 7.4, the slope of the curve turned to zero. Subsequently, 5 μm C_6-NBD-PC was added and the reaction catalyzed by PAF-AcH was monitored (**Fig. 11**). The experiment was also conducted with BAL fluid samples positive for PLase A_2 and PAF-AcH instead of the authentic enzymes. The same results were obtained when the activities were determined simultaneously in the same sample or in separate preparations. The component of the curve representing the activity of PLase A_2 was monitored for a longer period of time as its activity in BAL fluid was lower than that of the authentic enzyme (see Fig. 7). The enzymic activities were calculated from the slopes of the linear components of the curve.

To investigate the behavior of the fluorescence intensities of the standard C_6-NBD-FA and C_{12}-NBD-FA under these experimental conditions, 0.5 nmole of each NBD-FA was added successively to the 1-ml incubation buffer containing 5 nmoles C_6-NBD-PC, 5 nmoles C_{12}-NBD-PC, and 500 μg BAL fluid protein. As shown in Fig. 5, a remarkable linearity of the fluorescence was obtained. The A_{475} of the reaction mixture reached a value of 0.250 at the final incubation mixture.

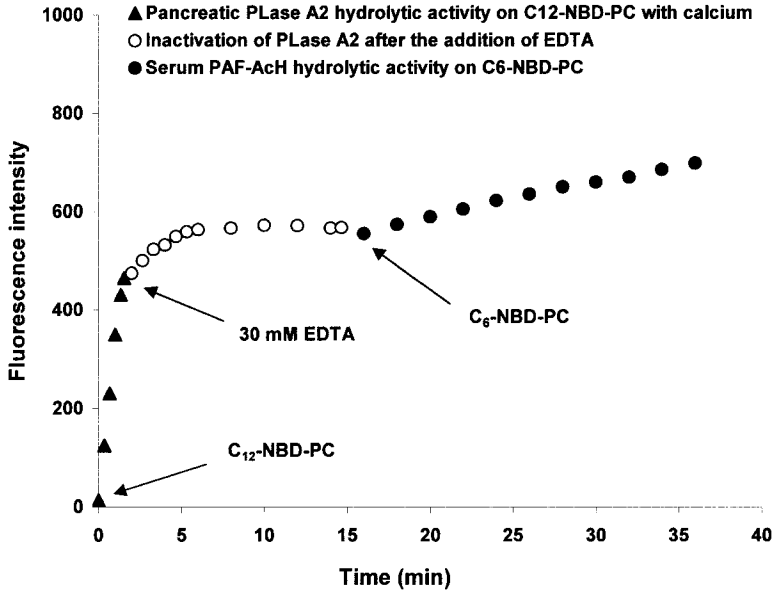

Fig. 11. Simultaneous determination of porcine pancreatic PLase A_2 and serum PAF-AcH. The incubation buffer contained 10 mm Tris-HCl, 2 mm Ca^{2+}, pH 7.4, 2 μg porcine pancreatic PLase A_2 and 100 μl of serum, diluted 1:50. The reaction started with the addition of 5 μm C_{12}-NBD-PC and was monitored directly with continuous excitation. Depletion of Ca^{2+} was achieved with 30 mm EDTA final concentration, at pH 7.4. After the slope of the curve had turned to zero, 5 μm C_6-NBD-PC was added and the reaction due to PAF-AcH was monitored.

DISCUSSION

In the present work C_6-NBD-PC and C_{12}-NBD-PC were evaluated as substrates for the investigation of endogenous to BAL PLase A_2 and PAF-AcH activities. It was found that C_6-NBD-PC in the absence of Ca^{2+} was hydrolyzed only by serum-PAF-AcH and not by porcine pancreatic PLase A_2. C_{12}-NBD-PC, in the presence of Ca^{2+}, was hydrolyzed only by PLase A_2. Based on this property, it was possible to determine the individual activities of PLase A_2 and PAF-AcH or to determine both enzymic activities differentially within the same sample.

The investigation of PLases A_2 in BAL fluid can provide information on the nature of a local insult in the lungs, where different forms of the enzyme, with preference to long-chain phosphatidylcholines, platelet-activating factor or even oxidized phosphatidylcholines, may coexist. It is known that phospholipases directly affect the function of lung surfactant (30, 31). Under specific situations like hypoxia, edema, local pH conditions, or Ca^{2+} concentration, not only the secretion of PLases A_2 by cells, but also their enzymic activities might be affected. In certain cases, like ARDS and pulmonary embolism, lyso-PC was detected (10, 19). The detergent properties of this phospholipid could contribute to the pathogenesis or perpetuation of the disorders (32).

Although NBD-PE species as substrates have been reported to enhance the sensitivity of the determination of synovial, type II PLase A_2 activity (23), NBD-PC species were used in this work, because BAL fluid contains PC as a major phospholipid component and the results were expected to be more representative.

In the first series of experiments, the fluorescence properties of the authentic substrates and reaction products under the specific experimental conditions, were examined. Above their CMC, pure suspensions of NBD-PC species exhibited low levels of fluorescence, possibly due to the self-quenching of the micellar structures. The fluorescence emitted by the authentic NBD-FAs was significantly greater than that of the corresponding NBD-PC species, allowing their quantitative determination (21). This increase was attributed to the monomer formation of NBD-FAs in the aqueous buffer solutions (33). The CMC of C_6-NBD-PC was found to be 0.2 μm. The CMC of C_{12}-NBD-PC could not be determined with the fluorimetric method applied, probably because of the methods' insensitivity. However, it should be lower than 0.2 μm because of the higher lipophilicity of C_{12}-NBD-PC in comparison to C_6-NBD-PC. Therefore, our experiments were conducted above the CMCs of both NBD-PCs. These results are in agreement to those of other investigators (21–23).

Inner filter effect is known to perturb the linear response of fluorescence as a function of a fluorophore's concentration when the absorbance of the reaction mixture at the excitation wavelength exceeds a certain limit. Referring to the experiments of the separate determination of PLase A_2 or serum PAF-AcH, the A_{475} did not exceed 0.100. Furthermore, there was a linear response of fluorescence versus concentration of NBD-FAs at least within the method's detection range. When larger concentrations of the fluorophores than usual were used, the linearity remained unaffected. In the simultaneous determination of both enzymic activities, although the A_{475} was around 0.250, the linear response of the NBD-FAs was not affected.

BAL fluid, serum diluted 1:50, BSA, or porcine pancreatic PLase A_2, in the absence of any fluorescence substance, did not emit fluorescence at 535 nm when excited at 475 nm. Therefore, light scattering did not interfere with our measurements.

The emission spectra of the NBD-PCs and the relevant NBD-FAs exhibited the same emission maxima in the presence of BAL fluid up to 500 μg protein/ml of incubation mixture or serum diluted 1:50. This ensures the accuracy of the measurements under our experimental conditions. However, for BSA concentrations higher than 100 μg protein/ml, C_{12}-NBD-FA but not C_6-NBD-FA manifested a shift in the emission maximum, with a concomitant increase in the fluorescence intensity. This effect is probably due to the higher lipophilicity of C_{12}-NBD-FA but it did not constitute a limitation to the accurate determination of the enzymic activities in BAL fluid or serum samples. The accuracy of the measurements could, in any case, be confirmed by internal standards application.

In concentrations above the CMC, the increase in fluorescence intensity of C_6- and C_{12}-NBD-PC suspended in the buffer solution was low and increased linearly in response to the substrate concentration. Similarly, the fluorescence increased linearly with the concentration of both authentic NBD-FAs, displaying the same slope. This, combined with the fact that both NBD-FAs emit at the same wavelength 50-fold more than the corresponding NBD-PCs, without shift in the emission maximum even in the presence of BAL fluid or serum, allows their simultaneous determination.

The fluorescence intensity of authentic NBD-PCs suspended in the buffer solution was not affected by pH variations. In the presence of BAL proteins, however, at pH 3.5 and before the initiation of the reaction, a remarkable increase in the fluorescence of the reaction mixture was observed. This could be due to an alteration in the micellar structures of the substrates that reduced the fluorescence quenching. At pH values in the neutral and alkaline region, the fluorescence intensity of both the NBD-FAs, did not change. Therefore, the direct monitoring of the reaction, without prior purification of the products, was feasible in that range. In the acidic region, however, the fluorescence intensity of C_{12}-NBD-FA decreased gradually, while that of C_6-NBD-FA remained unaffected. An explanation could be the fact that in acidic pH values, the solubility of C_{12}-NBD-FA in aqueous environments decreases. However, BAL fluid or serum samples can never have acidic or alkaline pH values. The fact that at low pH values the background fluorescence of NBD-PCs in the presence of BAL protein increases indicates that under such assay conditions the application of an internal standard could be useful.

The rise of temperature from 25°C, where the experiments were conducted, to 37°C, representing the op-

timal temperature for PLase A_2, resulted in a decrease of the emitted fluorescence for all the NBD-lipids examined, depending on the nature of the compounds. This phenomenon is observed with all the fluorescent molecules, as the augmentation of collisions might induce a loss of energy.

Sonication was applied to investigate whether the composition of BAL fluid affected the fluorescence as a physical magnitude, on the one hand, or the velocity of the reaction on the other, causing non-uniform background fluorescence alterations in BAL samples with different composition. It is known that phospholipases act preferentially on lipid–water interfaces. BAL fluid provides such a favorable environment, with lipids organized in various structures, like multi- or mono-lamellar bodies, vesicles, monomers etc., containing various amounts of proteins (9). Sonication of authentic C_6-NBD-PC in the buffer solution resulted to a 20% increase of the fluorescence intensity, while that of C_{12}-NBD-PC or NBD-FAs did not change. Sonication probably contributed to a better dispersion of C_6-NBD-PC, due to its higher polarity in comparison to the other NBD-lipids. Therefore, sonication is not desirable, as it contributes only to the augmentation of the background fluorescence.

In summary, to achieve reproducible results, given that the buffer's chemical composition does not pertain to the reactions' fluorescence, the pH value, the sonication conditions, if applied, and the incubation temperature should be kept constant. Under certain conditions, the application of an internal standard could be useful.

The fluorescence intensity of the different NBD-PCs varied unevenly in response to protein concentration. This, however, did not interfere with the accuracy of the method, as it represented the background fluorescence and was subtracted from all the measurements. Addition of BSA in the incubation buffer resulted in an increase in background fluorescence of both the substrates. This increase was low and proportional within a narrow range of BSA concentration in the buffer solution for C_6-NBD-PC and C_{12}-NBD-PC, respectively. This effect was probably due to the binding of lipids with BSA, which disturbed their micellar structures. The addition of BAL fluid in the incubation mixture also affected the substrates' background fluorescence in a similar manner. It should be mentioned that BAL fluid samples from patients without a lung disease contain up to 100 μg protein/ml of BAL fluid. In extreme conditions though, total protein content might reach high values, varying from 0.5 to 5.0 mg protein/ml of BAL fluid (10). Thus, the linear increase of background fluorescence as a function of protein concentration could reduce the detection limit of the reaction products and consequently the method's sensitivity. When BAL fluid was included in the incubation mixture instead of BSA, the linearity of the fluorescence enhancement of NBD-PCs was extended to a wider range of total protein concentration, at least up to 500 μg BAL fluid protein per ml of reaction mixture. This protein concentration is extremely high for BAL fluid samples, as mentioned above, and corresponds to 5 mg protein/ml BAL fluid. The effect of proteins on the background fluorescence could be due to the fact that different proteins, and particularly albumin which binds to lipids, may affect the fluorescence intensity of each NBD-PC in a different manner. Concerning the reaction products, the presence of BAL fluid and serum did not affect the fluorescence of either NBD-FA, despite their albumin content. Therefore, the determination of PAF-AcH and PLase A_2 activities using C_6-NBD-PC and C_{12}-NBD-PC, respectively, as substrates in samples containing BSA albumin is accurate.

In the absence of Ca^{2+}, authentic porcine pancreatic PLase A_2 was totally inactive, as expected. In the presence of Ca^{2+} the enzymic activity was positively correlated to the chain length at the *sn*-2 position of the PC species. Assuming that PLase A_2 exhibited 100% of its enzymic activity when [^{14}C]dipalmitoyl PC was the substrate, the activity with C_{12}-NBD-PC was satisfactory in the range of 80%, but with C_6-NBD-PC, enzymic activity dropped to 20%. Under our experimental conditions [^{3}H]PAF did not constitute a suitable substrate for porcine pancreatic PLase A_2. On the contrary, serum PAF-AcH in the absence or in the presence of Ca^{2+} was not capable of cleaving [^{14}C]dipalmitoyl-PC or C_{12}-NBD-PC, but it exhibited 50% of its activity against C_6-NBD-PC, considering as 100% the activity of PAF-AcH towards [^{3}H]PAF. Consequently, if C_6-NBD-PC was used as a substrate for the determination of PLase A_2 activity in samples with high PAF-AcH activity, like serum or BAL fluid (10), this could lead to an overestimation of PLase A_2 levels. This could possibly explain the discrepancies in results obtained by radiometric and fluorimetric PLase A_2 determination reported by other investigators (34).

In conclusion, using the fluorescent C_6-NBD-PC and C_{12}-NBD-PC it was possible to differentiate between PLase A_2 and PAF-AcH activities. Both activities could be measured in the same sample. Initially, PLase A_2, in the presence of 2 mm Ca^{2+} and using C_{12}-NBD-PC was determined, followed by Ca^{2+} depletion and addition of C_6-NBD-PC for the determination of PAF-AcH. In the presence of elevated protein content, C_6-NBD-FA could be used as an internal standard.

The method is reproducible and accurate, as it has been confirmed after purification of the products and evaluated after comparison with radiometric methods. It is low cost, convenient for direct monitoring, and lacks radioactivity hazards. It can be utilized as a screening method for samples such as BAL fluid or serum, potentially containing different PLase A_2 activities with preference for long and short *sn*-2 acyl chains.

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