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A fluorogenic phospholipid for the detection of lysosomal phospholipase A2 activity

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Abstract

Lysosomal phospholipase A2 (group XV PLA2, LPLA2) is a lysosomal enzyme linked to drug induced phospholipidosis. We developed phospholipid “smart probes” based on the conversion of a quenched fluorogenic substrate to a fluorescent product. Due to the preference of LPLA2 for phosphatidylglycerol, three fluorogenic phosphatidylglycerols were synthesized. Two fluorogenic phosphatidylglycerols were conjugated with one fluorescein amidite (FAM) group and one 4-(4-dimethylaminophenylazo)-benzoyl (DABCYL) group; the third substrate consisted of two FAM groups conjugated at the *sn*-1 and *sn*-2 positions. The *sn*-1 ester linkage was replaced with an amide linkage. 1-FAM-2-DABCYL-PG was degraded by recombinant LPLA2 and mouse serum but not by the serum obtained from LPLA2-deficient mice when 1,2-dioleoyl-PG/1-FAM-2-DABCYL-PG liposomes were used. The formation of 1-FAM-lyso-PG generated from 1-FAM-2-DABCYL-PG in the presence of LPLA2 was quantitatively determined by fluorescent measurements. The 1-FAM-2-DABCYL-PG incorporated into 1,2-dioleoyl-phosphatidylcholine/sulfatide liposomes was used to evaluate the effect of the cationic amphiphilic drugs amiodarone and fluoxetine on LPLA2 activity. The IC₅₀s of amiodarone and fluoxetine estimated by fluorescent measurement were 10 and 19 μM. These results indicate that 1-FAM-2-DABCYL-PG is a specific substrate for LPLA2 and a useful reagent for the detection of LPLA2 activity from multiple sources.

Keywords

phospholipase A2; phosphatidylglycerol; lysosome; phospholipidosis; cationic amphiphilic drug; amiodarone; fluoxetine; fluorophore

Introduction

Lysosomal phospholipase A2 (group XV phospholipase A2) is ubiquitously expressed in mammalian cells and tissues [1]. Phagocytic cells, most notably macrophages, highly express LPLA2. In these cells, the lipase degrades and clears intracellular and extracellular materials containing glycerophospholipids [2, 3]. The alveolar macrophages of three month old LPLA2 knock-out mice develop a foam cell phenotype consistent with phospholipidosis [3]. This phenotype is also observed in cells exposed to cationic amphiphilic drugs,

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suggesting that drug-induced phospholipidosis may result from the inhibition of LPLA2 activity [4, 5]. LPLA2 is a secreted protein as well as a lysosomal protein [6]. Thus, the intrinsic LPLA2 activity of tissues may be reflected by measuring the LPLA2 activity in extracellular fluids. Recently, we reported a specific detection method of LPLA2 activity in plasma and serum [7]. The method was also used to detect the LPLA2 activity in the aqueous humor from pigs [8].

However, this method, based on thin layer chromatography (TLC), is labor intensive, particularly if there are multiple samples to assay. Several fluorogenic phospholipids have been developed and been used as substrates for the detection of phospholipase A2 activity using either microscopy or a microplate reader [9]. However, these substrates are limited in the detection of LPLA2 activity of crude enzyme sources such as plasma and serum because of their lack of substrate specificity. Therefore, a need exists for the development of a LPLA2 specific fluorogenic phospholipid.

LPLA2 has a preference for anionic glycerophospholipids [10]. In the present study, we chose phosphatidylglycerol (PG) as the basis of new fluorogenic phospholipids. To limit the deacylation at the *sn*-2 position of PG, the ester linkage at the *sn*-2 position of PG was replaced by an amide linkage, and thus protected from phospholipase A catalysis. In addition, FAM and DABCYL were used as a fluorescent dye and a non-fluorescent quencher, respectively. In principle, individual phospholipids containing these groups are self-quenching, rendering the direct fluorescent measurement of LPLA2 activity possible if either functional group is released from the parent compound. On this basis, three novel fluorogenic compounds, 1-FAM-2-DABCYL-PG, 1-DABCYL-2-FAM-PG and 1,2-bis-FAM-PG, were designed and synthesized.

Recombinant LPLA2 and mouse serum were used as an enzyme source of LPLA2. We first investigated whether these new fluorogenic PGs act as a specific substrate of LPLA2. We then established conditions for the fluorescent measurement of the reaction product by a microplate reader. Finally, because PLA2 activity is inhibited by cationic amphiphilic drugs (CADs) such as amiodarone [10, 11], the assay system using a specific fluorogenic substrate to LPLA2 was applied to confirm the effect of CADs on LPLA2 activity.

Materials and Methods

Reagents

1,2-Dioleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (DOPG), 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine (DOPC), and *N*-acetyl sphingosine (NAS), were obtained from Avanti Polar Lipids Corp. (Alabaster, AL); amiodarone and fluoxetine were from Sigma-Aldrich (St. Louis, MO); recombinant mouse LPLA2 was from Proteos (Kalamazoo, MI); high performance thin layer chromatography silica gel plates, 10×20 cm, were from Merck (Darmstadt, Germany). Fluorogenic PGs, 1-DABCTL-2-FAM-PG, 1-FAM-2-DABCYL-PG and 1,2-bis-FAM-PG, were synthesized at Echelon biosciences (Salt Lake City, UT) and are now commercially available. Sulfatide was previously prepared in our laboratory.

Transacylase activity of LPLA2

The reaction mixture consisted of 49 mM sodium citrate (pH 4.5), 10 µg/ml BSA, 38 µM *N*-acetyl-sphingosine incorporated into 127 µM DOPG liposomes, and mouse serum or recombinant mouse LPLA2 in a total volume of 500 µl. The reaction was initiated by adding recombinant LPLA2, incubated at 37°C and terminated by adding 3 ml of chloroform/methanol (2:1, v/v) plus 0.3 ml of 0.9 % (w/v) NaCl. The mixture was centrifuged at 800g for 5 min at 20°C. The resultant lower organic layer was transferred into another glass tube and dried down under a stream of nitrogen gas. The dried lipid was dissolved in 40 µl of

chloroform/methanol (2:1, v/v), applied on a high performance thin layer chromatography plate and developed in a solvent system consisting of chloroform/acetic acid (9:1, v/v). The plate was dried and soaked in 8 % (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 6.8 % (v/v) H_3PO_4 , 32 % (v/v) methanol. Then the uniformly wet plate was briefly dried using a hair dryer and charred for 15 min in a 150°C oven. The plate was scanned and the content of the product (1-*O*-acyl-NAS) was estimated by NIH-ImageJ 1.37v.

LPLA2 activity using fluorogenic PGs

Thin layer chromatography based assay—The reaction mixture contained 49 mM sodium citrate (pH 4.5), 10 $\mu\text{g/ml}$ BSA, liposomes (130 μM phospholipid) and 28 ng/ml of recombinant mouse LPLA2 or 2% (v/v) mouse serum in 500 μl of total volume. The liposomes consisted of DOPG/fluorogenic-PG (20:1, molar ratio). The reaction was initiated by adding 10 μl of 0.9% NaCl, 1.45 $\mu\text{g/ml}$ recombinant mouse LPLA2 or mouse serum, kept at 37°C and terminated by adding 3 ml of chloroform/methanol (2:1, v/v) plus 0.275 ml of 0.9% NaCl. In addition, 50 μl of 0.5 N HCl was added to the mixture to recover some polar reaction products (anionic products) such as FAM-conjugated fatty acid and DABCYL-conjugated fatty acid into the organic phase. The resultant organic phase was dried down under a stream of nitrogen gas and applied to an HPTLC plate as described above. The plate was developed in a solvent system consisting of chloroform/acetic acid (9:1, v/v) or chloroform/methanol/acetic acid/water (60:16:2:1, v/v). DABCYL-conjugated fatty acid was detected as a red spot after drying the plate. FAM-conjugated fatty acid was detected as a fluorescent spot under UV-ray.

Fluorescent measurement by microplate reader—DOPG/1-FAM-2-DABCYL-PG (100:1, molar ratio) and DOPC/sulfatide/1-FAM-2-DABCYL-PG (100:10:1, molar ratio) liposomes were incubated with recombinant mouse LPLA2 as described in TLC assay. After terminating the reaction, the upper layer containing the reaction product, 1-FAM-lyso-PG, was transferred into to a small vial and alkalized with 6 N NaOH. The concentration of NaOH was adjusted to 0.1 N. Two hundred μl of the alkalized solution was put into a well of a 96-well black plate. The fluorescent intensity of the solution was measured by a microplate reader (SpectraMax M5, Molecular Devices LLC, USA). The emission and excitation wavelengths were 525 nm and 495 nm, respectively.

Results

LPLA2 activity against fluorogenic phospholipids

The molecular structures of new three fluorogenic PGs are shown in Fig. 1. Thin layer chromatography was employed to evaluate the reaction products produced from individual fluorogenic PGs in LPLA2 reaction. The reaction products were partitioned with chloroform/methanol/aqueous solution (2:1:0.8, v/v) under acidic conditions. The distribution of the reaction products in the organic phase is dependent on their *pK_a*, hydrophilicity, and hydrophobicity. Under these partitioning conditions, lyso-FAM-PG and lyso-DABCYL-PG were not recovered in the organic phase.

The degradation of 1-FAM-2-DABCYL-PG, 1-DABCYL-2-FAM-PG and 1,2-bis-FAM-PG by recombinant mouse LPLA2 under acidic conditions was confirmed by measuring the DABCYL-conjugated fatty acid released from 1-FAM-2-DABCYL-PG by densitometry (Fig. 2A, top) and FAM-conjugated fatty acid released from 1-DABCYL-2-FAM-PG and bis-FAM-PG by thin layer chromatography under UV detection (Fig. 2A, middle and bottom). A non-fluorescent chromophore of DABCYL-conjugated fatty acid was detected as a red spot on the plate. By contrast, the fluorophore of FAM-conjugated fatty acid was detected as a fluorescent spot appearing green on the plate under UV exposure. In addition,

the sera from wild type mice degraded these fluorogenic PGs under the same reaction conditions (Fig. 2B). The sera from LPLA2-deficient mice released FAM-conjugated fatty acid from 1-DABCYL-2-FAM-PG and bis-FAM-PG. However, the release of FAM-conjugated fatty acid from bis-FAM-PG by the sera of LPLA2-deficient mice was less than that released by the sera of wild type mice (Fig. 2B). In contrast to recombinant mouse LPLA2 or serum from wild type mice, the DABCYL-conjugated fatty acid released from 1-FAM-2-DABCYL-PG by the sera of LPLA2-deficient mice was extremely low (Fig. 2B). This specific reaction became further evident by using the liposomes with a higher ratio of 1-FAM-2-DABCYL-PG (Fig. 2C). Taken together these results indicate that 1-FAM-2-DABCYL-PG specifically acts as a specific substrate of LPLA2 under acidic conditions.

Fluorescence measurement of LPLA2 activity using a fluorogenic specific substrate, 1-FAM-2-DABCYL-PG

First, 1-FAM-2-DABCYL-PG was incorporated into DOPG liposomes. To avoid a physicochemical effect of the fluorogenic PG on the liposomes, the molar ratio of DOPG to 1-FAM-2-DABCYL-PG was adjusted to 100:1. In the LPLA2 assay system under acidic conditions, incubation of DOPG/1-FAM-2-DABCYL-PG liposomes with recombinant LPLA2 did not result in any fluorescent emission signal from the reaction mixture. The reaction product, 1-FAM-lyso-PG, produced from 1-FAM-2-DABCYL-PG by LPLA2 reaction was completely recovered in the upper layer after phase partition with C/M/Aqueous solution (2:1:0.8, v/v) of the reaction mixture. By contrast, another product, DABCYL-conjugated fatty acid, and 1-FAM-2-DABCYL-PG were completely recovered in the lower layer. Thus only 1-FAM-lyso-PG was the fluorophore found in the upper layer.

The fluorescent intensity of fluorescein in FAM is pH-dependent and is enhanced under basic conditions. To evaluate LPLA2 activity by formed 1-FAM-lyso-PG, the upper layer obtained after termination of the reaction was collected into a small vial and alkalized with NaOH as described in the materials and methods section. Two hundred μ l of the alkaline mixture was transferred into a well of a 96-well black plate and measured with a microplate reader. To quantify the content of 1-FAM-lyso-PG released by LPLA2, the fluorescence intensity of various known amounts of 1-FAM-lyso-PG was examined (Fig. 3A). Known amounts of liposomes containing 1-FAM-2-DABCYL-PG were hydrolyzed in the presence of 0.5 N NaOH. The ester linkage but not the amide linkage of 1-FAM-2-DABCYL-PG was cleaved by this alkaline treatment. The 1-FAM-lyso-PG produced from the liposomes in the presence of NaOH was extracted by chloroform and methanol as described in the materials and methods section. The fluorescence intensity of 1-FAM-lyso-PG recovered in the upper layer was measured by a microplate reader. A linear increase of lyso-1-FAM-PG was observed corresponding to 32 pmol (Fig. 3A). Six hundred forty pmol of 1-FAM-2-DABCYL-PG was contained in the reaction assay system, indicating that the initial velocity of LPLA2 activity could be measured with sufficient sensitivity and reliability using 1-FAM-2-DABCYL-PG.

The fluorescent intensity at 525 nm following excitation at 495 nm increased with time when DOPG/1-FAM-2-DABCYL-PG liposomes were incubated with recombinant mouse LPLA2 (Fig. 3B). Additionally, when 1-FAM-2-DABCYL-PG was incorporated into DOPC/sulfatide liposomes in the LPLA2 assay the sensitivity was greater than that observed when 1-FAM-2-DABCYL-PG was incorporated into DOPG liposomes not containing sulfatide (Fig. 3B). Furthermore, the formation of 1-FAM-lyso-PG in the presence of LPLA2 under acidic conditions increased linearly as a function of time (Figs. 3B and 4A) and of LPLA2 concentration (Fig. 4B) when DOPC/sulfatide/1-FAM-2-DABCYL-PG liposomes were used. These results support the conclusion that the fluorescence assay using 1-FAM-2-DABCYL-PG is a sensitive and quantitative measurement of LPLA2 activity.

The measurement of LPLA2 activity in the presence of cationic amphiphilic drugs using 1-FAM-2-DABCYL-PG

To evaluate the effect of CADs on LPLA2 activity, DOPC/sulfatide/NAS and DOPC/sulfatide/1-FAM-2-DABCYL-PG liposomes pre-incubated with 0, 1, 3.33, 10, 33.3 and 100 μM amiodarone and fluoxetine and then incubated with recombinant mouse LPLA2. Both amiodarone and fluoxetine inhibited LPLA2 activity in a concentration dependent manner (Fig. 5). The IC_{50} s of amiodarone and fluoxetine estimated from the fluorescent measurement were 10 μM and 19 μM , respectively (Fig. 5B). Using the transacylation method and DOPC/sulfatide/NAS liposomes to measure LPLA2 activity, the IC_{50} s of amiodarone and fluoxetine were 8.3 μM and 14.5 μM , respectively (Fig. 5A).

Discussion

Several kinds of fluorogenic phospholipids are commercially available. However, these phospholipids are hydrolyzed not only by phospholipase A2s but also by phospholipase A1s, lipases and esterases. In this study, we sought to characterize a new fluorogenic that is specific for LPLA2 activity.

LPLA2 has a preference for anionic glycerophospholipids [10], in particular PG. As shown in figures 1C and D, three individual fluorogenic PGs based on the chemical structure of PG were synthesized. All fluorogenic PGs were degraded by wild-type mouse serum as well as recombinant mouse LPLA2 (Fig. 2). Interestingly, unlike 1-DAB-2-FAM-PG and 1,2-bis-FAM-PG, 1-FAM-2-DABCYL-PG was poorly degraded in the presence of serum obtained from LPLA2-deficient mice. This implies that DABCYL-conjugated acyl chain introduced at C2 position of 1-FAM-2-DABCYL-PG is specifically recognized by LPLA2. The basis for the substrate specificity between LPLA2 and the 1-FAM-2-DABCYL-PG is unknown. However, the present results show that 1-FAM-2-DABCYL-PG is a highly specific in its recognition by LPLA2. 1-FAM-2-DABCYL-PG was therefore pursued as a promising basis for a fluorescence-based LPLA2 assay.

In spite of the hydrolysis of 1-FAM-2-DABCYL-PG by LPLA2, we could not directly observe the fluorescent emission of the reaction product, 1-FAM-lyso-PG, produced during the reaction. FAM contains a pH-dependent fluorophore, fluorescein. Fluorescein is protonated at pH less than 7, resulting in decreased fluorescence. The pK_a of fluorescein is 6.4. Therefore, it is difficult to detect fluorescence originating from FAM in the acidic reaction mixture (pH 4.5). Based on the phase partitioning of the reaction products under acidic conditions, only one of the reaction products, 1-FAM-lyso-PG, was recovered into the upper layer. This suggested that a quantitative fluorescence assay of LPLA2 activity using a microplate reader would be possible if the solution collected from the upper layer after lipid extraction of the reaction mixture was adjusted to a pH greater than 7. Under this condition the solution containing the reaction product, 1-FAM-lyso-PG, would fluoresce. This proved to be the case. As shown in figures 3 and 4, the reaction product recovered into the upper layer was quantitatively detected after adjusting the solution of the upper layer to an alkaline pH.

Sulfatide incorporated into DOPC/NAS liposomes enhances LPLA2 activity under acidic conditions. The enhancement by sulfatide is linear up to a 10% molar ratio of sulfatide to DOPC and weakened by the addition of NaCl in a concentration dependent manner [10]. Amiodarone, a CAD that interacts with negatively charged lipids and zwitterionic phospholipids [12], also reduced the LPLA2 activity against DOPC/sulfatide/NAS liposomes in a concentration dependent manner. These findings suggest that LPLA2 interacts with the anionic lipid membrane containing substrate through an electrostatic interaction. To confirm that the 1-FAM-2-DABCYL-PG based LPLA2 assay system works

by a similar mechanism, LPLA2 activity measurements using DOPC/sulfatide/NAS liposomes and DOPC/sulfatide/1-FAM-2-DABCYL-PG liposomes were compared in the presence of two representative CADs, amiodarone and fluoxetine. The IC_{50} s of amiodarone and fluoxetine estimated from fluorescent measurement were 10 μ M and 19 μ M, respectively, when DOPC/sulfatide/1-FAM-2-DABCYL-PG liposomes were used in the assay of LPLA2 activity. Both IC_{50} 's values were slightly higher than those obtained from the transacylation measurement using DOPC/sulfatide/NAS liposomes. 1-FAM-2-DABCYL-PG is an anionic phospholipid. A potential explanation for this difference is that DOPC/sulfatide/1-FAM-2-DABCYL-PG liposomes are presumed to be more negatively charged than DOPC/sulfatide/NAS liposomes in the assay conditions. Stated differently, the neutralization of the surface charge of the liposome membranes consisting of DOPC/sulfatide/1-FAM-2-DABCYL-PG requires more CAD than for DOPC/sulfatide/NAS liposomes without 1-FAM-2-DABCYL-PG. Therefore, the slight shift of the IC_{50} to higher concentrations for these CADs using the DOPC/sulfatide/1-FAM-2-DABCYL-PG might be the result of the augmentation of the negative charge of the liposomes.

Some of CADs that inhibit LPLA2 activity have been reported to induce phospholipidosis in mammalian cells and tissues [4]. Recently, in vitro assay for phospholipidosis-induced CADs using fluorescent phospholipid accumulation in a human monocyte cell line and HepG2 cell cultured in a 96-well plate has been reported [13, 14]. For CAD-induced phospholipidosis associated with LPLA2, the present assay system consisting of recombinant LPLA2 and liposomes containing 1-FAM-2-DABCYL-PG may provide a more effective and facile means to determine whether newly or already developed CADs evoke a LPLA2 mediated phospholipidosis.

In this study, we showed that a new fluorogenic phospholipid, 1-FAM-2-DABCYL-PG, serves as a highly specific substrate in LPLA2 activity assay under acidic conditions and is a potentially effective and useful probe to screen the compounds for LPLA2 inhibition by CADs. In practical terms, the elimination of the requirement for thin layer chromatography is a substantial improvement in the determination of LPLA2 activity. A new probe with a pH-independent fluorophore (FAM) is being developed. This improvement would potentially allow for the real time measurement of LPLA2 activity without lipid extraction and separation.

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1-DABCYL-2-FAM PG

1-FAM-2-DABCYL PG

1, 2-Bis-FAM PG

Fig. 1.
Fluorescent phosphatidylglycerol (PG) probe structures, including 1,2-bis-FAM-PG, 1-FAM-2-DABCYL-PG, and 1-DABCYL-2-FAM-PG.

Fig. 2.

A. Degradation of fluorogenic phospholipids by recombinant mouse LPLA2. The reaction was carried out as described in the text and kept for 2.5, 5, 10 and 20 min at 37°C. B. Degradation of fluorogenic phospholipids by the serums obtained from wild type mouse and from LPLA2-deficient mouse. The reaction was kept for 90 min at 37°C in the presence or absence of 2% mouse serum. 1-FAM-2-DABCYL-PG, 1-DABCYL-2-FAM-PG and 1,2-bis-FAM-PG denote the respective liposomes. C. Degradation of 1-FAM-2-DABCYL-PG by the serums obtained from wild type mouse and from LPLA2-deficient mouse. The reaction was carried out as described under “Materials and Methods” using DOPG/1-FAM-2-DAB-PG (10:1, a molar ratio) liposomes and kept for 45 and 90 min at 37°C in the presence or absence of mouse serum.

Fig. 3.

Fluorescence measurement of LPLA2 activity using 1-FAM-2-DABCYL-PG. A. To obtain the standard curve of 1-FAM-lyso-PG (shown in panel A), known amounts of liposomes containing 1-FAM-2-DABCYL-PG were hydrolyzed in the presence of 0.5 N NaOH for 1 h at 37°C. After the reaction, the reaction mixture was neutralized with HCl. The reaction products such as 1-FAM-lyso-PG and other lipids were extracted as described in the materials and methods section. The fluorescence intensity of 1-FAM-lyso-PG recovered in the upper layer partitioned by adding chloroform, methanol and 0.9% NaCl was measured as described in the materials and methods section. B. The reaction by LPLA2 was carried out in the presence of liposomes (128 μ M phospholipid) and 7.25 ng/ml of recombinant mouse LPLA2 in 500 μ l of total volume as described in the materials and methods section and kept for 2, 4, 6, 8 and 10 min at 37°C after adding 5 μ l of 0.9% NaCl or 725 ng/ml of recombinant mouse LPLA2. DOPG/1-FAM-2-DABCYL-PG (100:1, molar ratio, ■) liposomes and DOPC/sulfatide/1-FAM-2-DABCYL-PG (100:10:1, molar ratio, ●) liposomes were used. The fluorescent intensity of the reaction product recovered in the upper layer partitioned by adding chloroform, methanol and 0.9% NaCl was measured as described in the materials and methods section. The released reaction product, 1-FAM-lyso-PG, plotted in the figures were calculated from [(the fluorescent intensity in the presence of LPLA2)-(the fluorescent intensity in the absence of LPLA2)]/slope of Fig. 3A at each time point. In both panel A and panel B, error bars indicate S.D. (n = 4).

Fig. 4.

Quantitative measurement of LPLA2 activity using 1-FAM-2-DABCYL-PG. In panel A, the reaction mixture contained 49 mM sodium citrate (pH 4.5), 10 μ g/ml BSA, liposomes (128 μ M phospholipid) and 7.25 ng/ml of recombinant mouse LPLA2 in 500 μ l of total volume. The liposomes consisted of DOPC/sulfatide/1-FAM-2-DABCYL-PG (100:10:1, molar ratio). The reaction was initiated by adding the LPLA2 and kept for 1, 2 and 4 min at 37°C. The released reaction product, 1-FAM-lyso-NAS, was determined as described in the legend of Fig. 3 and plotted against incubation time. In panel B, different concentrations of the LPLA2 were incubated with DOPC/sulfatide/1-FAM-2-DAB-PG liposomes for 2 min at 37°C in 500 μ l of 49 mM Na-citrate (pH 4.5). The released reaction product, 1-FAM-lyso-PG, plotted in the figure was calculated as described in the legend of Fig. 3. In both panel A and panel B, error bars indicate S.D. (n = 4).

Fig. 5.

Inhibition study of LPLA2 activity by CAD using 1-FAM-2-DABCYL-PG. DOPC/sulfatide/NAS (100:10:30, molar ratio) and DOPC/sulfatide/1-FAM-2-DABCYL-PG (100:10:1, molar ratio) liposomes were pre-incubated with 0, 1, 3.33, 10, 33.3 and 100 μM amiodarone (●) and fluoxetine (■) for 5 min at 37°C. The reactions and measurements were carried out as described in the materials and methods section and the legend of Fig. 4. In panel A, the reaction was kept for 10 min in the presence of DOPC/sulfatide/NAS liposomes (127 μM phospholipid) and 14.5 ng/ml of recombinant mouse LPLA2 in 500 μl of total volume. The transacylase activity of LPLA2 was determined by 1-*O*-oleoyl-NAS produced from DOPC/sulfatide/NAS liposomes. The formation of 1-*O*-oleoyl-NAS in the absence of CADs was linearly proportional to the incubation time till 10 min (data not shown). In panel B, the reaction was kept for 4 min in the presence of DOPC/sulfatide/1-FAM-2-DABCYL-PG (128 μM phospholipid) and 7.25 ng/ml of recombinant mouse LPLA2 in 500 μl of total volume. LPLA2 activity was determined by the fluorescence measurement of the reaction product, 1-FAM-lyso-PG, released from DOPC/sulfatide/1-FAM-2-DABCYL-PG liposomes. In both panel A and panel B, error bars indicate S.D. (n = 4).