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A Real-Time Fluorogenic Phospholipase A_2 Assay for Biochemical and Cellular Activity Measurements

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Summary

A fluorogenic analog of the PLA_2 substrate PC, named Dabcyl-BODIPY-PC, or simply DBPC, was synthesized with a fluorescence quencher (Dabcyl, 4-[(4-[*N,N*-dimethylamino]phenyl)azo]benzoic acid) in the *sn*-1 acyl chain and a BODIPY fluor in the *sn*-2 acyl chain. DBPC was recognized by $sPLA_2$ from each of the four sources examined (bee venom, human synovial fluid, cobra venom, and bovine pancreas). A dramatic and quantifiable fluorescence enhancement of DBPC occurred upon phospholipase digestion both in the presence and absence of excess PC. Both real-time and endpoint assays for PLA_2 were sensitive, consistent, and rapid. Thus, DBPC can be used as a sensitive fluorogenic probe for in vitro high-throughput screening assays for PLA_2 activation and inhibition and would expedite studies of PLA_2 in cellular signaling, in vitro screening for drug discovery, and subcellular localization of enzyme activity.

Introduction

Phospholipase A_2 (PLA_2) enzymes comprise a growing family of enzymes widely expressed in insects, reptiles, and mammals. The isozymes differ in substrate specificity, cofactor requirements, subcellular locations, and physiological functions [1]. All PLA_2 isozymes cleave the *sn*-2 acyl chain of a glycerophospholipid to produce two potential lipid mediators: a free fatty acid and a *lyso*-phospholipid. For example, the cleavage of phosphatidylcholine (PC) to *lyso*-PC affords a chemoattractant for circulating monocytes [2]. Arachidonate, frequently found in the *sn*-2 position of mammalian phospholipids and all mammalian phosphoinositides, is the key eicosanoid precursor for the production of thromboxanes, prostaglandins, and leukotrienes. Recently, phospholipase D and several PLA_2 isozymes have been implicated in the production of LPA, a potent mitogen that is overproduced in ovarian cancer and that stimulates tumor invasiveness and reduces the sensitivity of cancer cells to chemotherapy [3].

The majority of the PLA_2 enzymes fall into four categories. First, the so-called secreted forms, or $sPLA_2$s, comprise more than nine small molecular-weight (14 kDa) isozymes represented by groups I, II, V, and X [4]. Second, the cytosolic PLA_2 ($cPLA_2$) isozymes include at least three 85 kDa group IV enzymes [5]. Third, the intracellular PLA_2 ($iPLA_2$) isozyme is an 80 kDa calcium-independent $cPLA_2$ of group VI [6]. Finally, there are a number of other PLA_2 families with single isoforms; an example is platelet-activating factor acetyl hydrolase, which hydrolyzes the *sn*-2 acetate but is not involved in eicosanoid signaling events [7]. The categorization of PLA_2 isozymes depends on molecular weight, biological source, calcium requirement, and other biochemical properties. The four major types may be distinguished by group-specific assays capable of measuring hydrolysis with subnanogram quantities of enzyme [8]. Indeed, $sPLA_2$ forms can be either intracellular or secreted, are widely found in many mammalian tissues, and are common lytic components of insect and snake venoms. The $sPLA_2$ isozymes bear no sequence similarity with $cPLA_2$ [9], which exhibits an overwhelming preference for phospholipids that contain arachidonic acid (AA) in the *sn*-2 position [10–12].

$cPLA_2$s and $sPLA_2$s are considered signaling PLA_2s, which regulate the stimulus-induced arachidonate metabolism, which is linked in turn to the production of bioactive eicosanoids. $iPLA_2$ has a major role in the remodeling of phospholipids [6], and recent evidence suggests that it can also participate in lipid signaling. Group IIA PLA_2 has antibacterial activity and also functions as a modifier of tumor multiplicity [13, 14]. Both $iPLA_2$ inhibition and depletion of human $iPLA_2$ demonstrate that this enzyme plays a key role in lymphocyte proliferation [15]. In apoptosis research, the membrane lipids of the apoptotic and damaged cells are preferred substrates for the PLA_2s. A number of extracellular and intracellular PLA_2 isozymes are substrates of caspases [16–18].

The PLA_2 family of isozymes is complex, and as new isozymes are discovered, it becomes apparent that some functions were incorrectly correlated with a particular isoform. For example, the recent discovery of other small molecular-weight PLA_2s has drawn attention to some of the functions that were previously attributed to group IIA PLA_2s. Continued discovery of new PLA_2 isoforms and development of a systematic understanding of this enzyme family is important for the understanding of disease pathologies and for the success of basic research. For the identification of active enzymes, several analytical approaches are employed. Most methods of measuring enzyme activity utilize extraction and chromatographic separation of end products. These classical endpoint assays involve radio-HPLC or radio-TLC separation of radiolabeled lipids [19, 20]. Endpoint assays do not permit continuous monitoring of phospholipase activation in live cells and cannot be readily

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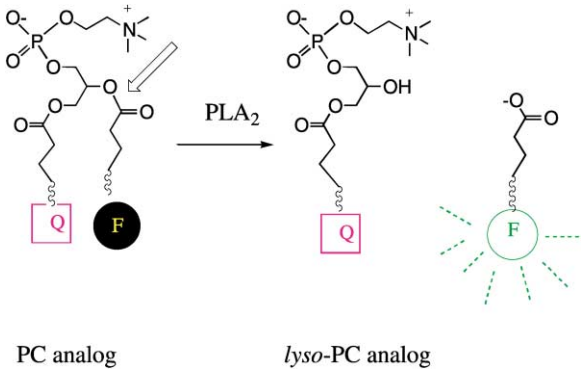

Figure 1. Principle of the Fluorogenic, or "Fluorescence Dequenching," Assay for PLA_2

The scissile ester bond is indicated by the open arrow.

applied to high-throughput screening for kinetic analysis or inhibitor discovery. The internally pi-stacked *bis*-BODIPY phosphatidylcholine (BBPC), a commercially available substrate for phospholipase A enzyme activity, has been used in continuous assays to monitor the responses of neutrophils [21]. However, BBPC applications were limited because incomplete intramolecular self-quenching of the fluorophores led to high background fluorescence with this probe. Moreover, BBPC assays show low sensitivity because only a modest enhancement of fluorescence occurred upon hydrolysis.

We now present the synthesis as well as the in vitro and in situ evaluation of a novel PC analog that allows monitoring of PLA_2 activity via a sensitive fluorogenic assay. The key concept is that of "fluorescence dequenching" (Figure 1), in which the internal quenching is provided by a nonfluorescent energy absorber rather than by the stacking of identical planar fluorophores as in BBPC. This new analog, Dabcyl-BODIPY-PC, or simply DBPC, has very low background fluorescence with distinct enhancement of the fluorescent signal when it is cleaved in both PS and cholesterol-PG-PC liposomes. This finding suggests its utility for in vitro screening for inhibitors of PLA_2. Finally, DBPC hydrolysis in the plasma membranes of canine kidney cells was observed after the induction of $cPLA_2$ activity. This finding provides a proof-of-concept for the development of a real-time cell-based assay for PLA_2 activity.

Results

The synthesis of 1-*O*-(6-Dabcyl-aminohexanoyl)-2-*O*-(6-[12-BODIPY-dodecanoyl]amino-hexanoyl)-*sn*-3-glycerylphosphatidylcholine (DBPC) (**7**) was carried out as shown in Figure 2. First, chiral *p*-methoxybenzyl (PMB)-protected glycerol was sequentially esterified with Fmoc (9-fluorenylmethoxycarbonyl) and *t*-Boc (*t*-butoxycarbonyl)-protected 6-aminohexanoic acids in the *sn*-1 and *sn*-2 positions, respectively, to give diester **2**. The PMB protecting group was then oxidatively removed, and intermediate alcohol **3** was phosphorylated with 2-chloro-2-oxo-1,3,2-dioxaphospholane [22] to give cyclic phosphate **4**. Reacting intermediate **4** with trimethylamine at 60°C gave the PC derivative **5**, in which concomitant Fmoc deprotection had occurred. Condensation of the primary amine of **5** with an activated ester containing the Dabcyl chromophore gave the *sn*-1 acyl derivative with the quencher installed. Finally, the *t*-Boc was removed to give intermediate amine **6**, which was condensed with BODIPY-succinimidyl ester to give the final product DBPC (**7**).

A molecular model of DBPC was constructed and analyzed with Cambridge Software Chem3D Pro. After energy minimization of DBPC, the conformation space was explored by molecular dynamics. Across a wide array of structures, the distance of BODIPY and Dabcyl (measured by the distance of F69 on BODIPY to N40 on Dabcyl) was in the range of 6–40 Å. Figure 3 illustrates two of the most stable conformations of DBPC, one of which has the BODIPY fluorophore partially pi-stacked with the Dabcyl chromophore.

The structure of DBPC is analogous to the structure of *bis*-BODIPY PC (BBPC, **8**) (see Figure 2), a commercially available material. In order to compare DBPC (**7**) and BBPC (**8**) as fluorogenic substrates, we performed endpoint hydrolysis experiments by using the commercially available PLA_2 isozyme from bee venom. The digestion of DBPC with *Apis mellifera* (bee) venom PLA_2 produced TLC spots for the fluorescent free fatty acid and a reddish colored Dabcyl *lyso*-PC spot (our unpublished data). After 1.5 hr of digestion of DBPC by bee venom PLA_2, the fluorescence enhancement approached 25-fold relative to the unhydrolyzed substrate (Figure 4A). In contrast, digestion of BBPC by bee venom PLA_2 for the same time period resulted in a modest 2.5-fold fluorescence enhancement. Under these experimental conditions, saturation was achieved at approximately 1.0 units (U) of bee venom PLA_2. The enhancement was largely due to the much lower intrinsic fluorescence of the DBPC substrate relative to that of BBPC.

Next, the substrate specificity of DBPC by $sPLA_2$ isozymes from a variety of biological sources was determined with a fluorescence microtiter plate reader. DBPC was a substrate for each of the four $sPLA_2$ sources studied: bee venom, human synovial fluid, bovine pancreatic fluid, and *Naja naja* (cobra) venom (Figure 4B); PS liposomes containing 10% of each fluorogenic compound were used. Among the four enzymes, bee venom and human synovial $sPLA_2$ were the most favorable enzymes for hydrolysis of DBPC.

Although DBPC, when applied to cell-based assays, may be subject to digestion by both PLA_1 and PLA_2, specific inhibitors can be used to differentiate the enzyme activities. For proof-of-concept studies, bee venom $sPLA_2$ was used to measure the kinetics of DBPC hydrolysis as well as inhibition with a specific $sPLA_2$ inhibitor, TEPC [23]. The fluorescence enhancement of the phospholipase reaction was recorded every 40 s in the absence of inhibitor, as well as for each of three inhibitor concentrations for a period of at least 20 min. The initial velocity was calculated from the first 2 min of the enzymatic reaction. A Lineweaver-Burk double-reciprocal plot was prepared from the kinetic data, and a K_M value of 18.0 μM for DBPC was calculated (Figure 5B). A Dixon plot (our unpublished data) replotting the slopes of the double-reciprocal lines for each inhibitor concentration afforded a K_I value of 0.9 μM for TEPC in

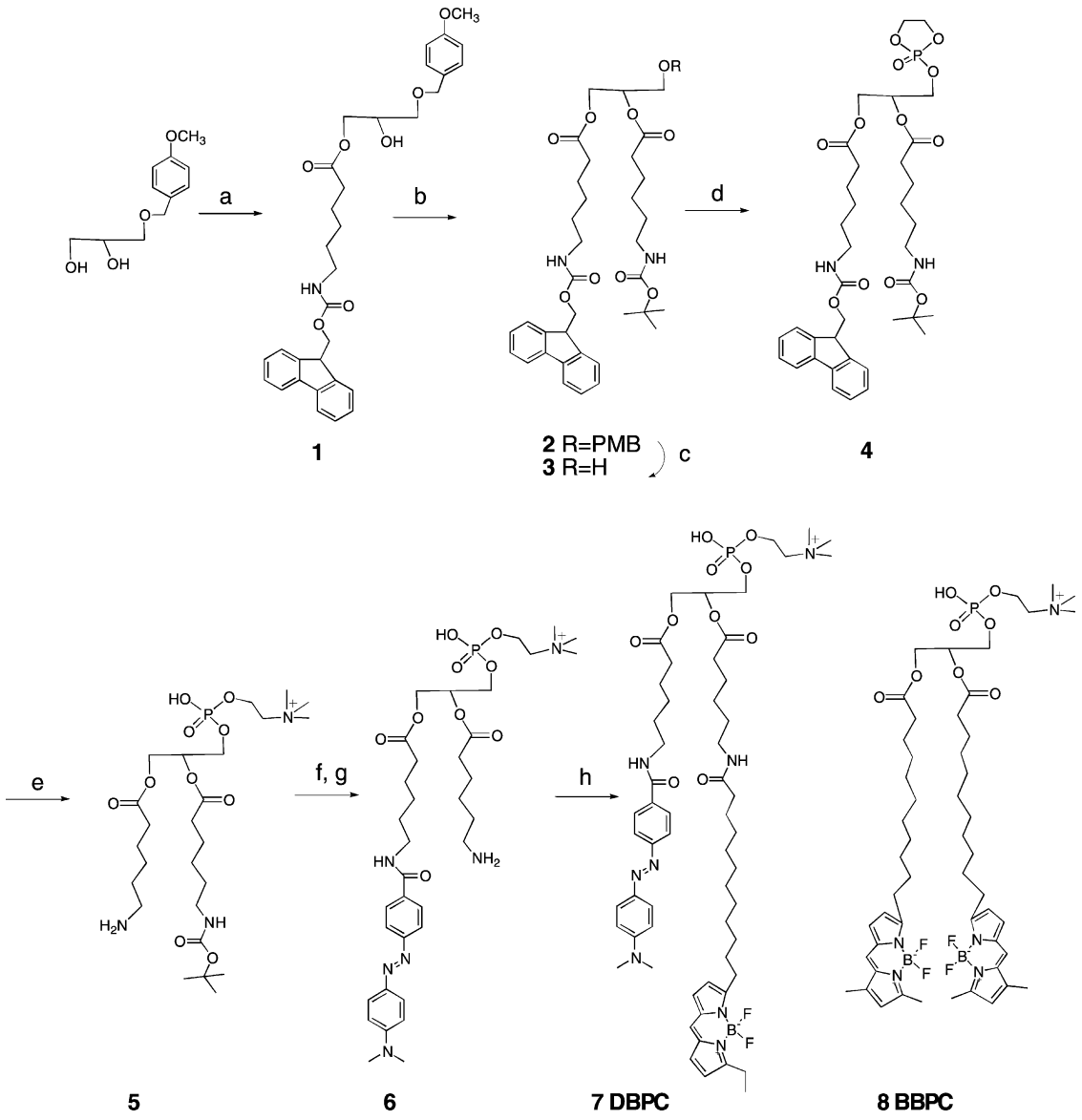

Figure 2. Synthesis of the Fluorogenic Substrate

Reaction conditions: (a) $FmocNH(CH_2)_5COOH$, DCC, DMAP, CH_2Cl_2, 4°C, overnight; (b) *t*-$BocNH(CH_2)_5COOH$, DCC, DMAP, CH_2Cl_2, r.t. overnight; (c) DDQ, CH_2Cl_2:H_2O (100:1), r.t., overnight; (d) $(CH_2)_2PO_3Cl$, Et(*i*Pr)$_2N$, toluene, r.t., overnight; (e) $(CH_3)_3N$, CH_3CN, 60°C, 20 hr; (f) Dabcyl-SE, DMF/TEAB (0.25 M, pH 8.0) (1:1), r.t., 5 hr; (g) TFA/CH_2Cl_2; (h) BODIPY-SE, DMF/TEAB (0.25 M, pH 8.0) (1:1), r.t., overnight.

this assay system. This result is comparable to the K_I value of 2 μM reported for cobra venom $sPLA_2$. To show that our DBPC assay system would also be applicable to human $sPLA_2$, we also examined the kinetic behavior of human synovial $sPLA_2$ with DBPC as well as its inhibition by TEPC. A K_M value of 14.2 μM was obtained for the human synovial $sPLA_2$, and TEPC showed an IC_{50} value below 2 μM for this enzyme (our unpublished data).

Next, we performed $sPLA_2$ assays in liposomes composed of cholesterol, PG, and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) (1:1:4 wt:wt:wt) and 20 wt% of BBPC or 20 wt% of DBPC in the liposome. These liposomes were sonicated and sized, and aliquots were placed on microtiter plates and analyzed with a fluorescence plate reader. Fluorescence changes were monitored in real time after the addition of aliquots of four concentrations of bee venom $sPLA_2$. It is noteworthy that both BBPC and DBPC were hydrolyzed in the presence of a >2.5-fold excess of competing PC in these assays. For BBPC, a 25% enhancement of fluorescence (over a high background) was observed at 35 min with 0.1 mg/ml $sPLA_2$, whereas with DBPC, enhancement greater than 300% was observed under the same conditions, with much lower background fluorescence (Figure 5A). Furthermore, DBPC-containing cholesterol-PG-PC liposomes were examined by confocal fluorescence microscopy in the absence (inset) and presence of cobra venom $sPLA_2$ (Figure 6).

Finally, a cell-based assay was performed with a bradykinin (BK)-inducible canine kidney cell line (MDCK-

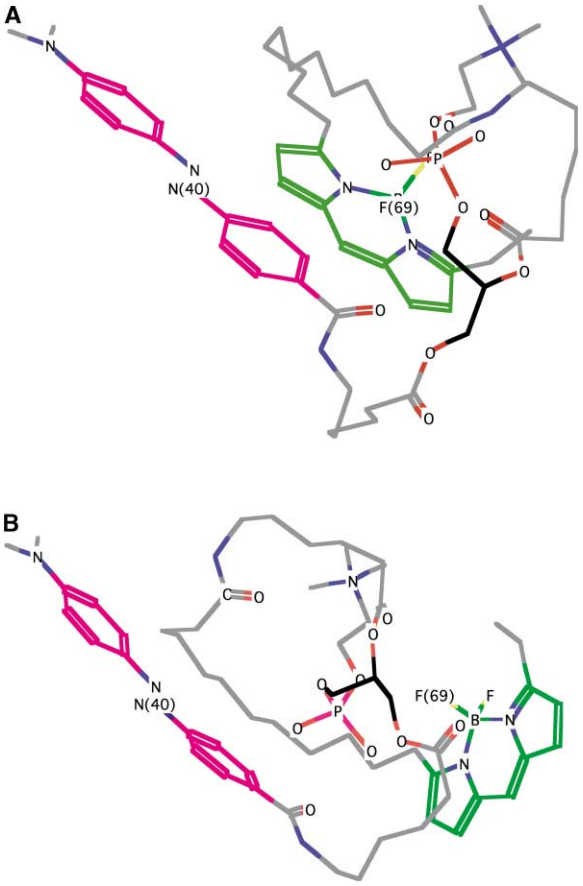

Figure 3. Two Maximally Stable DBPC Structures Obtained by Energy Minimization of Discrete Conformations after Molecular Dynamics

(A) F69-N40: 6.05 Å.
(B) F69-N40: 9.25 Å.

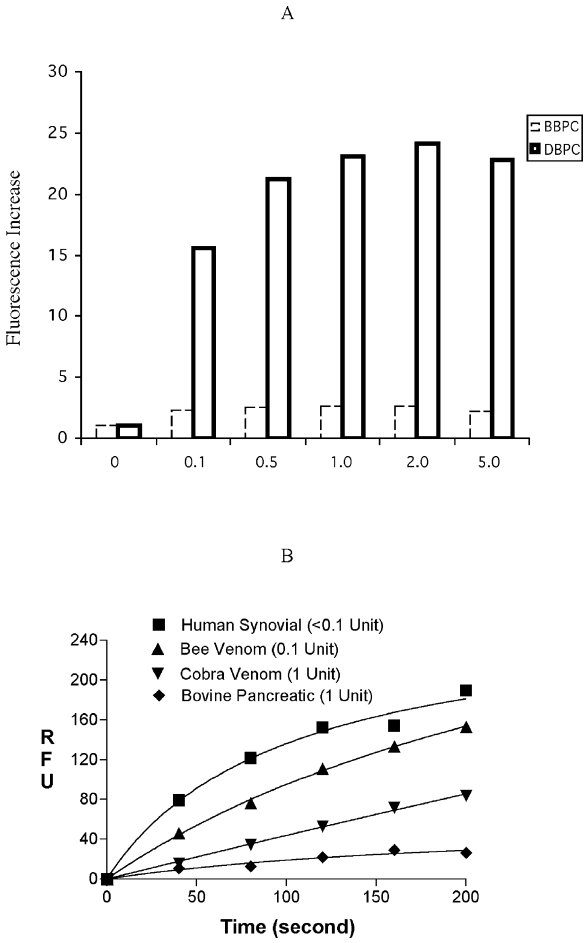

Figure 4. Hydrolysis of DBPC by $sPLA_2$s

(A) Fluorescence increase after hydrolysis of 8.86 μM (final volume = 100 μl) of either DBPC (**7**, solid bars) and BBPC (**8**, dotted bars) by bee venom $sPLA_2$ for 1.5 hr. Fluorescence enhancement is compared at different amounts of $sPLA_2$ from 0 to 5 U.
(B) Kinetic curves for digestion of DBPC **7** (4.84 μM in 100 μl final volume) with four secreted isozymes: human synovial $sPLA_2$ (<0.1 U, squares), bee venom (0.1 U, up triangles), bovine pancreas (1.0 U, diamonds), and cobra venom $sPLA_2$ (1.0 U, down triangles).

D1 cells) [24]. Cytosolic PLA_2 accounts for the majority of AA released from the *sn*-2 position of plasma-membrane PC. Thus, cells were treated with DBPC, which was passively incorporated into MDCK-D1 cell membranes. Cells were left without stimulation or were stimulated with 1 μM BK and monitored by confocal microscopy for 30 min. Figure 7 shows time zero and t = 3 min images for the unstimulated control cells (Figures 7A and 7B) and the BK-stimulated cells (Figures 7C and 7D). Cleavage of the *sn*-2 acyl chain by induced $cPLA_2$ released the fluorescent fatty acid, which appeared to remain predominately localized in the plasma membrane as a result of the hydrophobicity of the BODIPY fluorophore. No such fluorescence was observed in the plasma membrane of unstimulated cells.

Discussion

Because of high sensitivity and low cellular toxicity, the utilization of biochemical and biological assays based on fluorescence has increased dramatically. One of these methods employs fluorescence energy transfer between two fluorophores or between a fluorophore and a nonfluorescent chromophore (fluorescence quencher). We refer to fluorogenic assays in the latter category as "fluorescence dequenching" assays to distinguish them from fluorogenic assays in which a nonfluorescent chromophore becomes fluorescent when it is released from a substrate. An example of this type of fluorogenic assay is the liberation of fluorescein by a phosphatidylinositol-specific phospholipase C [25]. Fluorescence dequenching assays are desirable for real-time monitoring of enzymatic reactions in microtiter plate assays and in cell-based observations because they do not require isolation and separation of enzymatic products. Such applications have been applied to the monitoring of specific nucleic acid hybridizations with molecular beacons [26], for γ-glutamyl hydrolase [27], for DNA ligase [28], and at the completion of the work described herein, for PLA_2 [29–31].

The use of DBPC as a detection system for PLA_2 best demonstrates the principle of fluorescence dequenching. In an analogous fashion to BBPC, the Dabcyl group

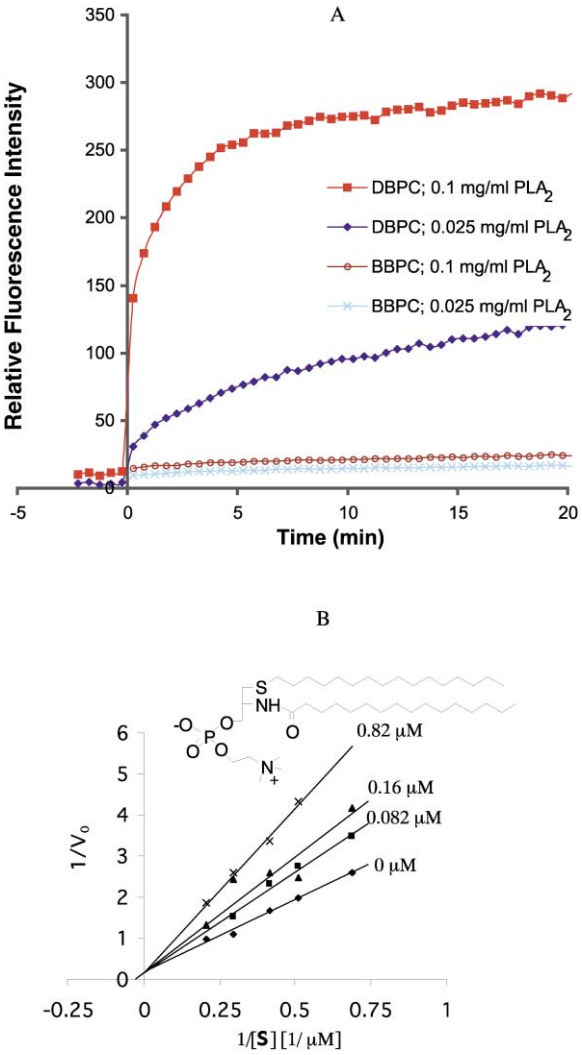

Figure 5. Kinetic Study of DBPC with Bee Venom $sPLA_2$

(A) Kinetics of fluorescence intensity increase of BBPC (20 wt%) or DBPC (20 wt%) in cholesterol-PG-PC (1:1:4) liposomes treated with 0.025 mg/ml or 0.1 mg/ml bee venom $sPLA_2$. Key: DBPC, 0.1 mg/ml PLA_2 (squares); DBPC, 0.025 mg/ml PLA_2 (diamonds); BBPC, 0.1 mg/ml PLA_2 (circles); BBPC, 0.025 mg/ml PLA_2 (x). For all data, background controls without PLA_2 were subtracted.

(B) Double reciprocal plot of the enzymatic reaction of bee venom $sPLA_2$ (0.05 U per 100 μl reaction) and DBPC with three different concentrations of the $sPLA_2$ inhibitor, TEPC. The calculated K_M for DBPC with the bee venom enzyme was 18.0 μM (our unpublished data). A Dixon plot of the slopes for three concentrations of TEPC gave a K_I value of 0.9 μM, compared with a K_I value of 2 μM for cobra venom [33].

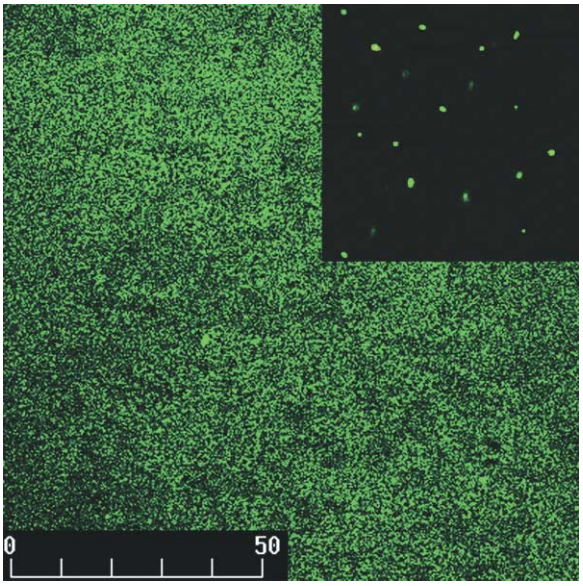

Figure 6. Fluorescence Micrograph of DBPC

Fluorescence micrograph of DBPC (20 wt%) in cholesterol-PG-PC (1:1:4) liposomes after 25 min treatment with 1 wt% cobra venom $sPLA_2$. The inset shows unstimulated liposomes. Scale bar = 50 μm.

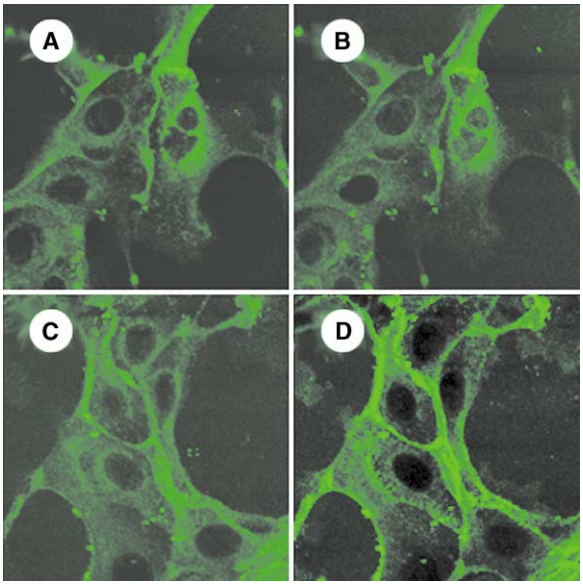

Figure 7. Activity of $cPLA_2$ in MDCK-D1 Cells

Activity of $cPLA_2$ in MDCK-D1 cells at t = 0 and 3 min in unstimulated (A and B) and BK-stimulated (C and D) cells. Hydrolysis was observed at the plasma membrane of cells with induced $cPLA_2$ activity.

of DBPC is in close proximity to and potentially pi-stacked with the BODIPY fluorophore [32]. Dabcyl can absorb a photon emitted by an excited BODIPY fluor but cannot fluoresce; it thus diminishes the background fluorescence of the substrate via intramolecular energy transfer. The background fluorescence of the DBPC substrate is thus substantially diminished relative to the pi-stacked two-fluor BBPC substrate. When the intramolecular relationship in DBPC is disrupted, e.g., by hydrolysis of either the *sn*-1 or the *sn*-2 acyl chain by either a PLA_2 or PLA_2 isozyme, the quencher and fluorophore become separated (Figure 1) and the BODIPY emission is no longer quenched. Figure 3 illustrates two of the most stable DBPC conformations, one of which has the BODIPY fluorophore nearly pi-stacked with the Dabcyl chromophore. The optimized molecular model of DBPC was obtained with a favorable BODPY-Dabcyl distance for resonance energy transfer. The usual distance for a fluorescence acceptor to interact with the

donor fluorophore is 10–100 Å. In the minimized models, the distance between the fluorescence donor and acceptor is in the low end of this range and explains the efficient intramolecular fluorescence quenching observed.

In our studies, the commercial two-fluor substrate BBPC was inferior to DBPC. BBPC uses one fluorophore to quench another fluorophore, which contributes to a much higher background. In elegant work, Farber and coworkers [30] reported monitoring PLA_2 activity in zebrafish embryos by using a new fluorescence dequenching probe similar to DBPC. This report, which appeared after we had completed the synthesis and evaluation of DBPC, employed a dinitrophenyl quencher in a modified phosphatidylethanolamine head group and a very short *sn*-2 acyl chain. This probe had the advantage that PLA_1 activity would not interfere with the fluorescence intensity but lacked convincing mimicry of the PC substrate. Detailed kinetic and in vitro enzymatic data were lacking in this report. A modified BBPC with two different fluors was also described, but background and differentiation of PLA_1 and PLA_2 activity were still not achievable. The approach of incorporating both the quencher and the fluor in different acyl chains, a synthetically more difficult challenge, had not yet been previously reported.

As described above, DBPC was recognized with significant signal-to-noise ratio by each of the four $sPLA_2$ isozymes examined, with the result that bee venom and human synovial PLA_2 showed the most favorable activity for this PC analog. Moreover, the amount of the human synovial enzyme used was only about 0.5 ng, suggesting that DBPC would be an excellent substrate for continuous monitoring of the activity of this enzyme in an assay for the discovery of therapeutic agents targeted at joint inflammation. Although the sensitivity of the fluorogenic assay was sensitive enough to permit the use of DBPC for cobra venom and bovine pancreatic $sPLA_2$ reactions, there was an order of magnitude lower efficiency in the processing of DBPC as a substrate by these two enzymes than by bee venom and human $sPLA_2$s. Given the known substrate selectivities of different PLA_2 enzymes, the differential activity of four $sPLA_2$ isozymes toward DBPC was not unexpected.

DBPC is also susceptible to other enzymatic reactions. Thus, we used an isozyme-specific inhibitor to confirm that the fluorogenic reaction indeed measured the fluorescence increase due to PLA_2 activity. Thus, the kinetics of hydrolysis of DBPC by bee venom and human $sPLA_2$ were examined with increasing amounts of TEPC as an inhibitor (Figure 6). TEPC is a competitive, reversible inhibitor of $sPLA_2$, with an IC_{50} value of 2 μM for cobra venom $sPLA_2$ [33, 34]. The K_I value of 0.9 μM for inhibition by TEPC of the hydrolysis of DBPC by bee venom $sPLA_2$ was fully consistent with the fluorescence increase being attributable to hydrolysis of the quenched PC analog by $sPLA_2$.

The hydrolysis of DBPC in cholesterol-PG-PC liposomes as shown in Figures 5A and 6 has two important implications for this study. First, it demonstrates that hydrolysis of the quencher-fluor modified PC analog can occur in a 2.5-fold excess of competing unmodified PC. Although this lowers the net enhancement measured (25-fold in PS liposomes versus 3-fold in PG-PC liposomes), it validates the potential for DBPC as an $sPLA_2$ substrate both in vitro and in situ.

Finally, we tested the utility of DBPC for the visualization of PLA_2 activity in MDCK-D1 cells. Insel and coworkers have used this cell line to exhaustively analyze BK-mediated activation of AA release and phosphoinositide hydrolysis [35]. Both the release of arachidonate from PC and phosphoinositide hydrolysis occur in response to the B1 and B2 receptor agonist BK. We observed that release of the fluorescent BODIPY-labeled fatty acid from DBPC was detectable in less than 1 min (our unpublished data) after treatment of cells with 1 μM BK and was sustained for more than 20 min. Importantly, most of the fluorescence increase was observed in the plasma membrane of the stimulated cells, suggesting that the BODIPY-fatty acid that was released remained in the membrane during the BK stimulation (Figure 7). These results differ significantly from those observed for MDCK-D1 cells loaded with BBPC prior to BK stimulation. With BBPC-loaded cells, the majority of the fluorescence increase was observed in intracellular bilayers (our unpublished data). Because both the fatty acid and the *lyso*-PC product contain identical BODIPY fluors, this result cannot be readily interpreted because the source of the fluorescence remains cryptic. These observations appear to suggest that DBPC accumulates primarily in the plasma membrane, whereas BBPC accumulates both in the plasma membrane and in other intracellular bilayers. Taken together, our results indicate that DBPC, in addition to providing a superior probe for in vitro measurements of the activity and inhibition of PLA_2, serves as a superior probe for subcellular localization of PLA_2 activity in cells.

Significance

A fluorogenic analog of the phospholipase A substrate PC was prepared by chemical synthesis. This analog, named DBPC, featured a Dabcyl quencher moiety in the *sn*-1 acyl chain and a BODIPY fluorescent moiety in the *sn*-2 acyl chain. The intramolecular proximity of the Dabcyl and BODIPY chromophores resulted in effective quenching of BODIPY fluorescence by intramolecular energy transfer in the DBPC substrate. Hydrolysis of DBPC by PLA_2 released the *sn*-2 acyl-linked BODIPY moiety, and fluorescence increased as a function of the extent of lipase activity. This "fluorescence dequenching" assay occurred with approximately 25-fold enhancement of fluorescence at a moderate level of hydrolysis of DBPC by bee venom PLA_2 in PS liposomes and a 3-fold enhancement in cholesterol-PG-PC liposomes. The large fluorescence increase was clearly due to the low intrinsic background of the substrate that normally exists in a fluorescence-quenched state. The substrate specificity of DBPC was examined against commercially available $sPLA_2$ isozymes. Bee venom $sPLA_2$ and human synovial $sPLA_2$ were the most favorable enzymes for DBPC hydrolysis, but $cPLA_2$ activity in BK-induced canine kidney cells also hydrolyzed DBPC in the plasma membrane, as visualized by confocal microscopy. The feasibility of using this probe for in vitro inhibition assays in microtiter plates

was demonstrated by measurement of the real-time kinetic behavior of DBPC and its inhibition by the specific $sPLA_2$ inhibitor TEPC. DBPC is thus a sensitive probe for a continuous fluorogenic assay applicable to both in vitro and cell-based in situ screening assays for PLA_2 activators and inhibitors.

Experimental Procedures

Synthesis of Fluorogenic Substrate

1*-O-*(6-Fmoc Aminohexanoyl)*-*3*-O-*(p-*Methoxybenzyl)*-sn-*Glycerol (1)

PMB-protected glycerol [36, 37] was reacted with 3.68 g of Fmoc-protected 6-aminohexanoic acid in the presence of dicyclohexylcarbodiimide (DCC) (2.33 g) and 4-*N,N*-dimethylaminopyridine (DMAP; 140 mg) in CH_2Cl_2 at 4°C. TLC analysis indicated that the reaction was complete after 12 hr. The reaction mixture was filtered, and CH_2Cl_2 was evaporated in vacuo. The residue was dissolved in ethyl acetate (EtOAc) and was filtered again. The condensed residual mixture was chromatographed twice on SiO_2, and elution with 20% EtOAc in dichloromethane gave a TLC-homogeneous product in 60% yield. R_f (50% EtOAc-hexane): 0.69. ^1H NMR ($CDCl_3$): δ 7.76 (*J* = 7.9 Hz, d, 2H, Fmoc), 7.58 (*J* = 7.3 Hz, d, 2H, Fmoc), 7.39 (*J* = 7.9 Hz, t, 2H, Fmoc), 7.30 (*J* = 7.9 Hz, t, 2H, Fmoc), 7.21 (*J* = 9.2 Hz, d, 2H, PMB), 6.85 (*J* = 8.6 Hz, d, 2H, PMB), 4.99–4.87 (m, 1H), 4.46 (s, 2H, CH_2O), 4.38 (*J* = 6.1 Hz, d, 2H, Fmoc), 4.26–4.05 (m, 2H), 4.05–3.93 (m, 1H), 3.75 (s, 3H, PMB), 3.54–3.36 (m, 2H), 3.23–3.07 (m, 2H), 2.41–2.25 (m, 2H), 1.71–1.54 (m, 2H), 1.54–1.41 (m, 2H), 1.40–1.20 (m, 2H) ppm. HRMS (FAB): calculated for $C_{32}H_{37}NO_7Na^+$ (M + Na^+), 570.2458; found, 570.2488.

1*-O-*(6-Fmoc Aminohexanoyl)*-*2*-O-*(6-*t-*Boc-Aminohexanoyl)*-*3*-O-*(p-*Methoxybenzyl)*-sn-*Glycerol (2)

Compound **1** (2.06 g) was stirred with 1.2 equivalents of *t*-Boc-6-aminohexanoic acid, 1.2 equivalents of DCC, and 0.1 equivalents of DMAP in CH_2Cl_2 for 48 hr at 4°C. The reaction was stopped by filtration of the dicyclohexylurea byproduct, and the crude mixture was purified on SiO_2 by elution with 50% EtOAc-hexane. The unreacted **1** was recovered, and 1.6 g of homogeneous diacyl derivative **2** was obtained (73% yield). R_f (50% EtOAc-hexane): 0.83. ^1H NMR ($CDCl_3$): δ 7.78 (*J* = 8.5 Hz, d, 2H, Fmoc), 7.60 (*J* = 8.5 Hz, d, 2H, Fmoc), 7.39 (*J* = 8.0 Hz, t, 2H, Fmoc), 7.31 (*J* = 8.5 Hz, t, 2H, Fmoc), 7.22 (*J* = 6.1 Hz, d, 2H, PMB), 6.86 (*J* = 6.6 Hz, d, 2H, PMB), 5.29-5.17 (m, 1H), 4.50–4.28 (m, 5H), 4.28–4.05 (m, 2H), 3.79 (s, 3H, OCH_3, PMB), 3.54 (*J* = 5.2 Hz, d, 2H), 3.26–3.02 (m, 4H), 2.37–2.21 (m, 4H), 1.69–1.57 (m, 4H), 1.58–1.30 (m, 17H) ppm. HRMS (FAB): calculated for $C_{43}H_{56}N_2O_{10}Na^+$ (M + Na^+), 783.3818; found, 783.3790.

1*-O-*(6-Fmoc Aminohexanoyl)*-*2*-O-*(6-*t-*Boc-Aminohexanoyl)*-sn-*Glycerol (3)

Compound **2** (400 mg) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (155 mg) were dissolved in wet (1% v/v) CH_2Cl_2, and the yellowish solution immediately turned green. After the solution was stirred overnight at room temperature (r.t.), TLC indicated that the reaction was complete. The reaction was diluted with CH_2Cl_2, and 10 ml of water was added. The water phase was extracted with 3 × 15 ml of CH_2Cl_2. The combined organic extracts were concentrated and purified on SiO_2 with 66% EtOAc-hexane. A pale yellow oil (240.5 mg) was obtained with a yield of 71.4%. R_f (60% EtOAc-hexane): 0.39. ^1H NMR ($CDCl_3$): δ 7.76 (*J* = 7.9 Hz, d, 2H, Fmoc), 7.59 (*J* = 7.9 Hz, d, 2H, Fmoc), 7.40 (*J* = 8.5 Hz, t, 2H, Fmoc), 7.32 (*J* = 7.9 Hz, t, 2H, Fmoc), 5.15–5.06 (m, 1H), 4.24–4.30 (m, 3H), 4.30–4.15 (m, 2H), 3.78–3.65 (m, 2H), 3.23–2.98 (m, 4H), 2.40–2.22 (m, 4H), 1.72–1.55 (m, 4H), 1.55–1.25 (m, 17H). HRMS (FAB): calculated for $C_{35}H_{48}N_2O_9Na^+$ (M + Na^+), 663.3245; found, 663.3289.

1*-O-*(6-Fmoc Aminohexanoyl)*-*2*-O-*(6-*t-*Boc-Aminohexanoyl)*-*3*-O-*(2-Chloro-2-Oxo-1,3,2-Dioxaphosphalyl)*-sn-*Glycerol (4)

Under an atmosphere of Ar, compound **3** (315 mg) was dissolved in 15 ml of toluene, and then diisopropylethyl amine (102.7 μl) was added. The solution turned yellow. Then, 2-chloro-2-oxo-1,3,2-dioxaphospholane (54.2 μl) [22] was added, and the reaction solution turned brown. The reaction was stirred 24 hr, another aliquot of phosphorylation reagent was added, and the reaction was stirred for an additional 24 hr. The solvent was removed in vacuo, and the residue was used without further purification.

1*-O-*(6-Aminohexanoyl)*-*2*-O-*(6-*t-*Boc-Aminohexanoyl)*-sn-*Glyceryl Phosphatidylcholine (5)

Crude compound **4** was dissolved in 2 ml of CH_3CN and cooled to −78°C. Then, $(CH_3)_3N$ was cooled on ice, and 0.5 ml of $(CH_3)_3N$ was poured into the reaction mixture [22]. The reaction was stirred at 60°C–65°C for 20 hr under a condenser charged with dry ice/acetone. Solvents were removed in vacuo, and an unsuccessful attempt was made to purify the crude material on SiO_2 by elution with CH_2Cl_2:MeOH:H_2O (10:5:1). In the final procedure, the crude PC derivative was used without further purification. The ^1H NMR indicated that the Fmoc group had also been removed during the conversion of the cyclic phosphate to the phosphocholine head group.

1*-O-*(6-Dabcyl-Aminohexanoyl)*-*2*-O-*(6-Aminohexanoyl)*-sn-*Glyceryl Phosphatidylcholine (6)

Compound **5** (crude, 100 mg) was dissolved in 0.25 M triethylamonium bicarbonate (TEAB) (4 ml), and 20 mg of Dabcyl-SE was added. After overnight reaction, TLC showed a new red spot at R_f = 0.3 (MeOH:$CHCl_3$:H_2O, 10:1:1). The mixed solvents were concentrated by a stream of air. The reaction residue was dissolved in MeOH:$CHCl_3$:H_2O (10:1:1) and purified on SiO_2 to give 15 mg of derivative **6**. ^1H NMR ($CDCl_3$): δ 7.92–7.78 (m, 6H, Dabcyl), 6.73 (*J* = 8.6 Hz, d, 2H, Dabcyl), 5.20–5.10 (m, 1H), 4.40–3.85 (m, 6H), 3.68–3.45 (m, 2H), 3.44–3.36 (m, 2H), 3.28–3.10 (m, 11H), 3.10–2.96 (m, 8H), 2.37–2.20 (m, 4H), 1.70–1.15 (m, 21H) ppm. ^{31}P NMR: δ 0.5 ppm. HRMS (FAB): calculated for $C_{40}H_{64}N_6O_{11}PNa^+$ (M − H + Na^+), 857.4175; found, 857.4214.

The compound was further treated with TFA (2 ml TFA:2 ml CH_2Cl_2). The solution immediately turned dark red upon the addition of TFA. After 2 hr, a single red spot appeared at R_f = 0.16 (MeOH:$CHCl_3$:H_2O, 10:1:1). TFA and CH_2Cl_2 were removed in vacuo, and the reddish residue was washed several times with ether to remove traces of TFA. The amine was used without further purification. HRMS (FAB): calculated for $C_{35}H_{56}N_6O_9P^+$ (M + Na^+), 735.3833; found, 735.3828.

1*-O-*(6-Dabcyl-Aminohexanoyl)*-*2*-O-*(6-(12-BODIPY-Dodecanoyl)Aminohexanoyl)*-sn-*Glyceryl Phosphatidylcholine (7)

Compound **6** (9.1 mg) was reacted with 8 mg of BODIPY-SE for 12 hr at r.t.; TLC showed a major reddish and fluorescent spot at R_f = 0.3 (MeOH:$CHCl_3$:H_2O, 10:1:1). The compound was purified on SiO_2 to give 2.8 mg of DBPC (**7**). ^1H NMR ($CDCl_3$): δ 7.99 (*J* = 8.0 Hz, d, 2H, Dabcyl), 7.90–7.78 (m, 6H, Dabcyl), 7.05 (s, 1H, BODIPY), 6.95 (*J* = 5.5 Hz, d, 2H, BODIPY), 6.74 (*J* = 9.2 Hz, d, 2H, Dabcyl), 6.26 (*J* = 6.2 Hz, t, 2H, BODIPY), 5.28–5.15 (m, 1H), 4.42–3.96 (m, 6H), 3.70–3.56 (m, 2H), 3.45 (s, 9H, -$N(CH_3)_3$), 3.30–3.10 (m, 4H), 3.08 (s, 6H, $N(CH_3)_2$), 3.03, 2.98 (*J* = 7.4 Hz, dt, 4H), 2.40–2.24 (m, 6H), 2.16–2.08 (m, 4H), 1.90–1.80 (m, 29H). ^{31}P NMR: δ 0.5 ppm. HRMS (MALDI): calculated for $C_{58}H_{87}BF_2N_8O_{11}P^+$: 1135.6323; found, 1135.6393.

TLC Analysis of DBPC and BBPC Cleavage by PLA_2

Aliquots (2 μg each) of DBPC and BBPC (Molecular Probes) were dissolved into 50 μl buffer (100 mM Tris [pH 8.0 for bovine pancreatic $sPLA_2$ or pH 8.9 for bee venom $sPLA_2$]) and sonicated three times at intervals of 1 min. Then PLA_2 (1 U) was added to the buffer, and the reaction was incubated at either 25°C (bee venom $sPLA_2$) or 37°C (bovine pancreatic $sPLA_2$). Hydrolysis was monitored by TLC (MeOH:$CHCl_3$:H_2O, 10:1:1).

Molecular Modeling

The structure of DBPC was constructed in ChemDraw Ultra 6.0, and the molecular model was obtained by going to "get 3D model" under the "scripts" menu. The model was first energy minimized by molecular mechanics (MM2 algorithm) in Chem3D 5.0. Molecular dynamics (MM2) was then carried out at a step interval of 2.0 fs and a target temperature of 298K. The conformations after each 10 ps were energy minimized, and these local energy-minimized structures were compared in terms of total energy and the distance between BODIPY (F69) and Dabcyl (N40).

Preparation of Phosphatidylserine (PS) Liposomes and Fluorogenic Enzyme Assay

DBPC or BBPC was combined with PS (bovine brain, 98% purity, Sigma Chemical Co. St. Louis, MO) (1:9) in $CHCl_3$ and the liposomes of DBPC/PS and BBPC/PS (80-100 μM) were prepared as described [21]. In each well of a 96-well plate with a non binding surface (Corning Incorporated Life Sciences, Acton, MA), the liposomes were incubated with aliquots of four different $sPLA_2$ isozymes (*Apis mellifera* (bee) venom, human synovial fluid, *Naja naja* (cobra) venom, and bovine pancreas (Sigma) and the fluorescence measurement (Molecular Devices Gemini XS, Sunnyvale, CA) was taken using either scanning, end-point, or kinetic modes. The data were collected in replicates and analyzed using an Excel spreadsheet.

Preparation of Cholesterol-PG-PC Liposomes and Fluorogenic Assay

Three liposome constituents (cholesterol, 1-α-phosphatidylglycerol (PG) and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) (Avanti Polar Lipids, Alabaster, AL) were suspended in chloroform and were mixed in an Eppendorf tube. The following quantities were used: 4.25 μl of 150 mg/ml cholesterol, 67 μl of 10 mg/ml PG, 264 μl of 10 mg/ml POPC, and 1 μg of BBPC (previously dried in a tube) or 6.25 μl of 160 μg/ml DBPC. This mixture, which contained 20% by weight of DBPC or BBPC and 80% by weight of a mixture of 1:1:4 cholesterol:PG-PC, was thoroughly mixed and then coated on the tube as a film by solvent evaporation under a stream of nitrogen. Then, 1.5 ml of 1 M Tris-HCl buffer (pH 8.9) was added, and the tube was sonicated with a Fisher Scientific 550 Sonic Dismembrator (setting 3, 45 s) to give a turbid, white suspension; sonication was continued (2 min, setting 3, ice-water bath) to clarify the mixture by formation of liposomes. The fluorescently labeled liposomes were observed by confocal microscopy.

The liposome mixture was aliquoted into 100 μl volumes in a 96-well plate, and measurements were taken with a FL600 Microplate Fluorescence Reader (BIO-TEK) that was set for kinetic readings (sensitivity = 65) and 15 s time intervals. The background fluorescence was recorded for 2 min, and then 10 μl of H_2O was added to the first row of wells (standards). Subsequently, 10 μl of bee $sPLA_2$ (0.025 and 0.10 mg/ml) was added to the remaining wells. The plate was then read on the kinetic setting for 30 min at intervals of 15 s. The data was plotted in Excel as changes in fluorescence intensity versus time, or percent increase versus time.

Fluorescence was also observed in the liposomal system with the confocal microscope. The liposomes were immobilized on a coverslip with 1-(3-[dimethylamino]propyl)-3-ethylcarbodiimide hydrocholoride (EDCI) and Sulfo NHS and then viewed as described below for the Madin-Darby canine kidney-D1 (MDCK-D1) cells.

Cell-Based Assay for DBPC Hydrolysis In Situ

MDCK-D1 cells (a gift of Professor P. Insel, University of California, San Diego) were cultured in DME-F12/Hams medium containing 7.5% equine serum and 2.5% calf serum (HyClone Laboratories, Logan, UT). The cells were passaged and trypsinized regularly to maintain 50% or less confluency. The MDCK-D1 cells were allowed to attach to sterile glass coverslips after trypsinization. Cells were then incubated in 100% Hank's Balanced Salt Solution (HBSS) (HyClone) for 30 min prior to labeling with fluorescent lipids, followed by imaging. The cells were incubated with 1 μg of DBPC resuspended in 30 μl of HBSS after being attached to a well apparatus that allowed for media manipulation and cell imaging at the same time. Cells were then washed with three 20 μl volumes of HBSS and viewed on a BioRad 1024 laser scanning confocal microscope (Richmond, CA) with a 60× objective lens. The BODIPY fluors were spectrally compatible with the krypton-argon laser excitation wavelength of 488 nm and filtered emission detection at 515 nm. After focusing and the recording of time-zero images, the cells were either stimulated with 1 μM BK (Sigma) or treated with a blank (for the unstimulated control). Images were saved from time intervals ranging from 1 to 25 min.

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