Synthesis of photoreactive phosphatidylethanolamine and its interaction with phospholipase A_2

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Abstract A photoreactive derivative of phosphatidylethanolamine, *N*-(4-azidobenzoyl)phosphatidylethanolamine (AB-PE), was synthesized by acylation of phosphatidylethanolamine with an *N*-hydroxysuccinimide ester of 4-azidobenzoic acid. The substantial photosensitivity exhibited by AB-PE correlated with a marked decrease in the absorption spectra of the compound. The compound proved sensitive to lipase and phospholipase A_2 hydrolysis but resistant to phospholipase C and D activities. Photolysis of a sonicated dispersion of AB-PE containing phospholipase A_2 resulted in irreversible inhibition of the enzyme. Addition of natural phosphatidylethanolamine provided protection against photoinactivation.—**Rajasekharan, R., and J. D. Kemp.** Synthesis of photoreactive phosphatidylethanolamine and its interaction with phospholipase A_2. *J. Lipid Res.* 1994. **35:** 45–51.

Supplementary key words azido-phospholipids • desaturation • photoaffinity label

Phospholipids play an important role in both the structure and function of membranes. It is now established that, in photosynthetic tissue, phosphatidylcholine (PC) (1, 2) and phosphatidylethanolamine (PE) (3) in the endoplasmic reticulum are substrates for desaturation of oleate to linoleate. In plants, the synthesized polyunsaturated fatty acids are made available for triacylglycerol biosynthesis in oilseeds. The key enzymes of triacylglycerol biosynthesis are membrane-bound and attempts to solubilize and purify them from plant sources have been largely unsuccessful. While there are a variety of methodologies that may be used to isolate membrane proteins, an alternative more direct approach involves covalent labeling of membrane proteins with site-directed ligands bearing chemically reactive, photoactivable groups. Azido-phospholipids have been demonstrated to be very effective photoprobes for studying a number of membrane-bound proteins, such as cytochrome b_5 (4), cytochrome c oxidase (5), PC-transfer proteins (6, 7), aminophospholipid transporter (8, 9), ATPase (10), gramicidin-A (11), and β-hydroxybutyrate dehydrogenase (12). These results encouraged us to synthesize azido analogs of PE as a first step in the isolation and purification of various PE and LPE-utilizing enzymes from oilseeds.

In the present study, we describe a simple method for synthesis of azido PE analogs. To study the efficacy of the synthesized analogs, phospholipase A_2 was targeted for specific covalent labeling. The analogs were efficiently used as substrate in the dark indicating that they bind to substrate binding site. Upon irradiation in the presence of the photoprobes, phospholipase A_2 activity was irreversibly inhibited and the inhibition was diminished by the addition of PE. These experiments suggest that the reactive nitrenes generated by photolysis from photoactive phospholipids may interact either at residues within active site or at the interfacial recognition site or possibly at both sites of phospholipase A_2.

MATERIALS AND METHODS

Materials

PE, PC, lysophosphatidylethanolamine (LPE), lysophosphatidylcholine (LPC) and *N*-(dinitrophenyl)phosphatidylethanolamine (DNP-PE) were purchased from Avanti Polar Lipids (Birmington, AL). *N*-Hydroxysuccinimidyl-4-azidobenzoate (HSAB) was obtained from Pierce (Rockford, IL). Lipase (*Rhizopus arrhizus*), phospholipase A_2 (*Crotalus adamanteus*), phospholipase C (*Clostridium perfringens*), phospholipase D (cabbage), acyl-CoA synthetase, acyl-CoA oxidase, and catalase were the products of Sigma (St. Louis, MO). All the reagents and solvents for chemical synthesis were obtained from Aldrich (Milwaukee, WI) as the highest grade available.

Abbreviations: AB-PE, *N*-(4-azidobenzoyl)phosphatidylethanolamine; DNP-PE, *N*-(dinitrophenyl)phosphatidylethanolamine; PC-I, 1-acyl-2-(12-azidooleoyl)glycerophosphorylcholine; PE-I, 1-acyl-2-(12-azidooleoyl)glycerophosphorylethanolamine; HSAB, *N*-hydroxysuccinimidyl-4-azidobenzoate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine.

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Synthesis and purification of *N*-(4-azidobenzoyl)phosphatidylethanolamine derivatives

N-(4-Azidobenzoyl)phosphatidylethanolamine was synthesized by the acylation of PE with the *N*-hydroxysuccinimide ester of 4-azidobenzoic acid. PE (25 mg, 33 μmol) was dissolved in 4 ml of dry benzene (containing 0.1 M triethylamine) and HSAB (9 mg, 35 μmol) was then added with constant stirring. After 12 h in the dark at room temperature, a 5-μl aliquot of reaction mixture was chromatographed on a silica thin-layer plate in chloroform–methanol–acetic acid–water 170:25:25:5. There were no ninhydrin-positive compounds detected after the plate was sprayed with ninhydrin reagent. The entire reaction mixture was completely dried under a stream of nitrogen and the residue was resuspended in 4 ml chloroform–methanol 1:1. Subsequent addition of 2 ml water and 1 ml chloroform resulted in the separation of two phases (13). The upper phase was discarded and the lower chloroform phase was evaporated to complete dryness under a stream of nitrogen. Residual water was removed from the residue by two successive additions and evaporations of benzene. The residue was dissolved in 2 ml chloroform and applied onto a silicic acid column (5 g). The column was washed with 50 ml chloroform and the product was then eluted with 50 ml chloroform–methanol 4:1 and dried under reduced pressure. Approximately 24 mg of AB-PE was obtained. The AB-PE was stored in chloroform at −80°C.

Preparation of *N*-(4-azidobenzoyl)lysophosphatidylethanolamine

AB-PE (5 mg) was converted to AB-LPE with phospholipase A_2. The enzyme was added in three 10-U portions at 1 h intervals. The substrate was completely hydro-

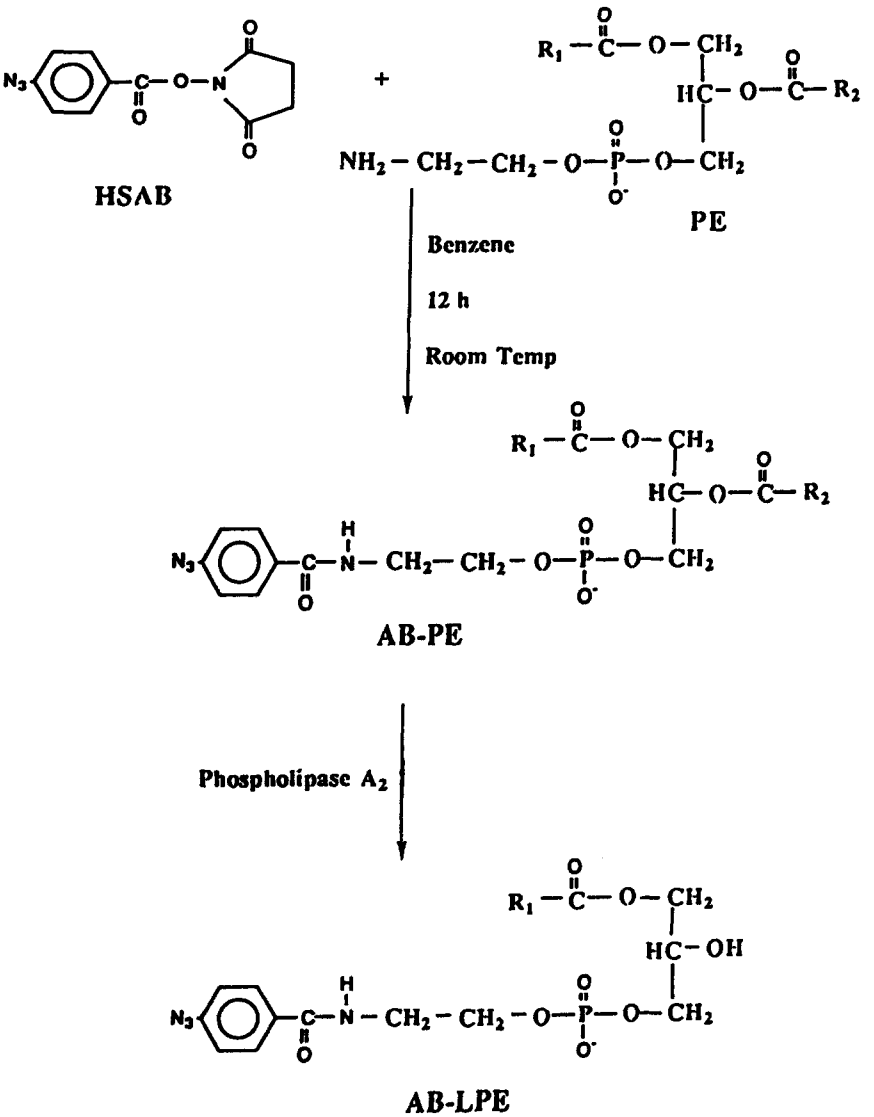

Fig. 1. Reaction scheme for the synthesis of *N*-(4-azidobenzoyl)phosphatidylethanolamine.

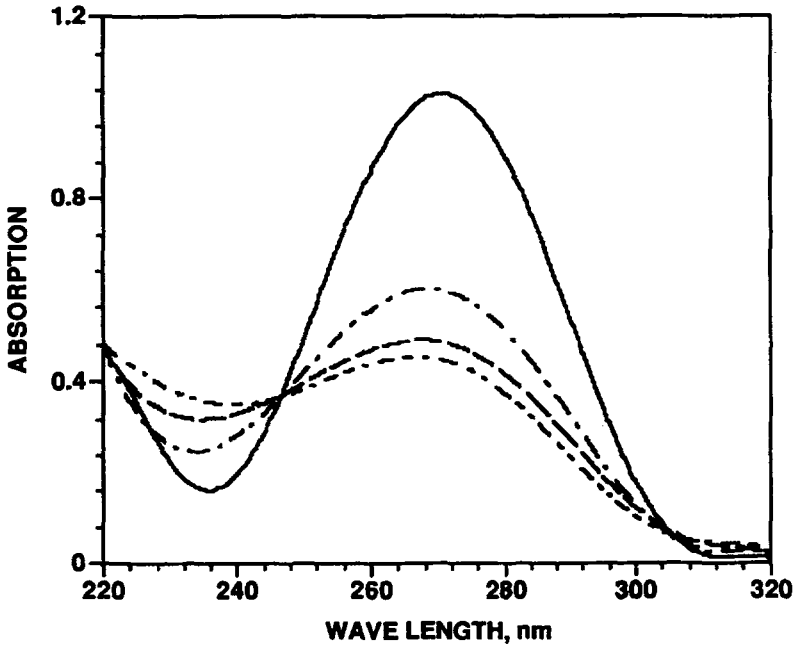

Fig. 2. Ultraviolet irradiation of AB-PE. Samples in ethanol were exposed to various times of irradiation and the spectra were taken. Irradiation time: (——) 0 s, (——·——·) 15 s, (—— ——) 30 s, (·····) 60 s.

lyzed at the end of 3 h. The purification was achieved simply by washing the insoluble Ca^{2+} salts of AB-LPE, formed during the reaction, with anhydrous ether. To avoid possible acyl migration from position-1 to position-2, the AB-LPE used in these preparations was not subjected to silicic acid column chromatography (14).

Synthesis and purification of 1-acyl-2-(12-azidooleoyl) glycerophosphorylcholine (PC-I)

The preparation was carried out essentially according to the procedure of Mason, Broccoli, and Huang (15). PC-I was synthesized by the acylation of LPC with 12-azidooleoyl anhydride using the catalyst 4-pyrrolidinopyridine. 12-Azidooleoyl anhydride was prepared by the reaction of 12-azidooleic acid (Sigma, St. Louis, MO) with dicyclohexylcarbodiimide in dry carbon tetrachloride (16). Commercially available LPC (10 mg, 22 μmol) was dissolved in 2 ml dry chloroform; 100 μmol azidooleoyl anhydride (63 mg) in 2 ml chloroform was then added with constant stirring followed by the addition of 25 μmol 4-pyrrolidinopyridine (3.7 mg). The mixture was shaken at 37°C for 5 h in the dark. Then the reaction mixture was then acidified with 0.1 N HCl and lipid was extracted. The lower chloroform phase was evaporated to dryness under a stream of nitrogen. The residue was resuspended in 2 ml chloroform and loaded onto a silicic acid column previously equilibrated with chloroform. The column was washed with chloroform to remove the unreacted fatty acid and then eluted with mixtures of chloroform and increasing amounts of methanol. PC-I eluted at a mixture of chloroform–methanol 1:1. Purity was established by thin-layer chromatography using the same solvent system as described above. The average yield was about 46%.

1-Acyl-2-(12-azidooleoyl)glycerophosphorylethanolamine was prepared from the PC analog by a phospholipase D-catalyzed base exchange reaction in the presence of excess ethanolamine (17). The PE analog was purified using silicic acid column chromatography as described earlier. The purity of these phospholipids and their azide analogs was routinely checked by silica gel thin-layer chromatography. All operations involving azides were performed in the dark or under dim light.

Phospholipase A_2 and lipase assay

The enzyme was assayed as described in detail by Kates (18). The phospholipids and their analogs (1 μmol) were dissolved in 1 ml ether–methanol 98:2 and the solution was stirred in the presence of 0.1 M Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$ and 10 U of phospholipase A_2 at 37°C for 30 min. The released fatty acid was extracted with anhydrous ether which was then evaporated under a stream of nitrogen. The amount of fatty acid was estimated by an enzyme-coupled, colorimetric method (19). The reaction mixture consisted of 50 mM Tris-HCl (pH 8.0), 0.5 mM CoA, 5 mM ATP, 7.5 mM $MgCl_2$, 0.1 mM FAD, and 0.3% Triton X-100. The reaction mixture was added to the fatty acid-containing tube giving a total volume of 200 μl. The mixture was sonicated followed by the addition of 50 μl acyl-CoA synthetase (0.1 U), 50 μl acyl-CoA oxidase (0.5 U), and 0.2 ml mixture of Ti(IV) and 4-(2-pyridylazo)resorcinol in a total volume of 0.5 ml.

The reaction was carried out for 20 min at 30°C. After incubation, the solution was cooled to room temperature and the absorbance was measured at 508 nm.

Phospholipase C and D assay

The enzymes were assayed quantitatively by estimating phospholipid without acid digestion by the method of Raheja et al. (20). The area containing phospholipid on the thin-layer chromatogram was scraped and used for estimation without eluting the phospholipid from silica gel. One ml chloroform and 0.2 ml chromogenic reagent were mixed and placed in a boiling water bath for 2 min. The tubes were cooled to room temperature and 2 ml chloroform was added. The contents were mixed and centrifuged. The lower blue organic layer was carefully removed and the absorbance was measured at 710 nm. PE was used as the standard.

Photolysis

Photolysis was carried out on ice in the dark for 10 min before exposure with a hand-held UV lamp with the filter removed (5000 microwatts/cm², model UVG-54, Ultraviolet Products, Inc., San Gabriel, CA) at a distance of 8 cm for 3 min. Azido-phospholipid was suspended in 25 mM Tris-HCl, pH 8.0, by sonication for 2 min. The photolabeling reaction was carried out in a final volume of 100 μl containing 5 U of enzyme in 25 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, and 100 μM sonicated dispersion

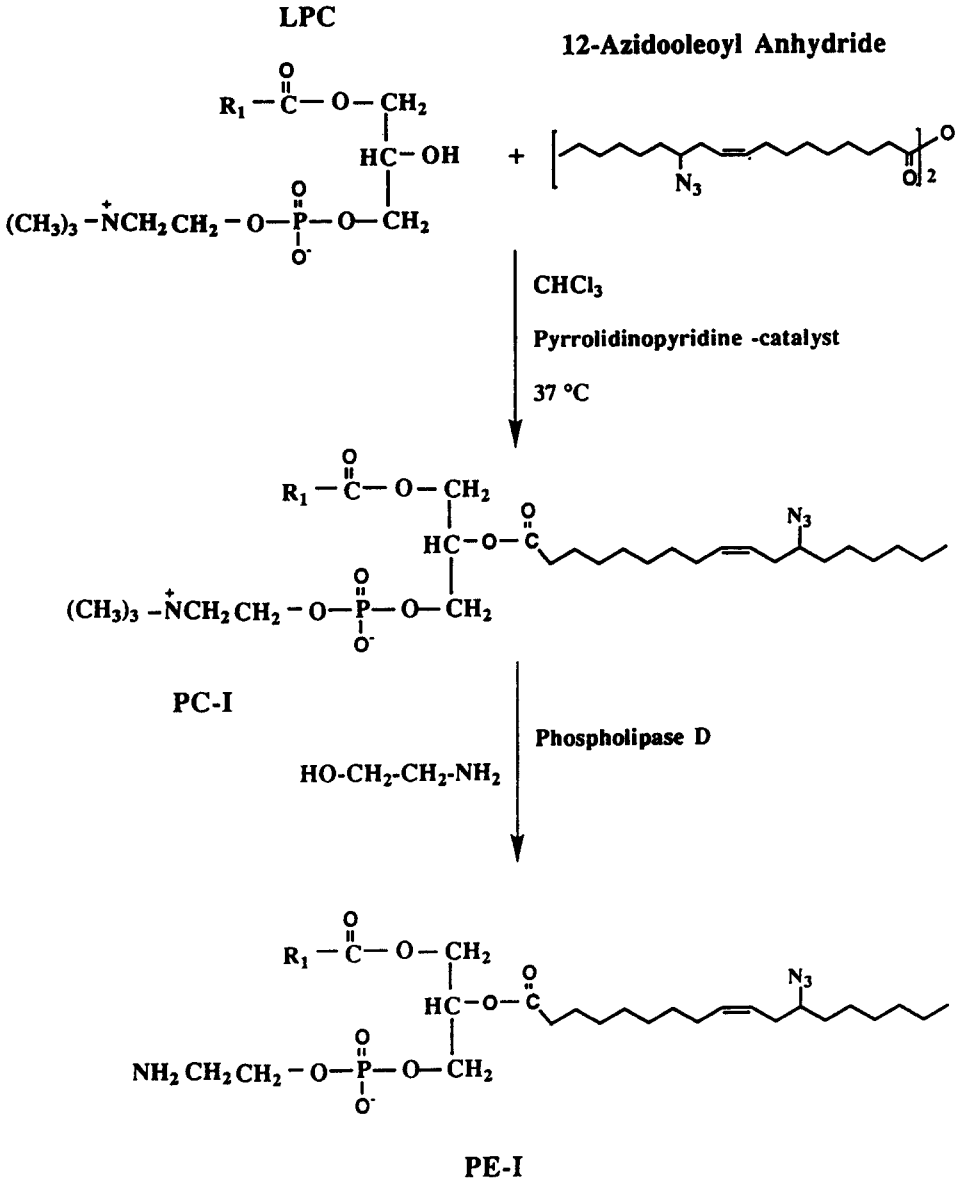

Fig. 3. Schematic representation of the synthesis of 1-acyl-2-(12-azidooleoyl)glycerophospholcholine and 1-acyl-2-(12-azidooleoyl)-glycerophosphoethanolamine.

of photoprobe followed by the addition of 20 μl of ether. As a control, the substrate was first exposed to UV light and then added to the enzyme. The labeling reaction was quenched by the addition of 10 μl of 100 mM DTT. The photolyzed samples were used directly for enzymatic assays.

RESULTS AND DISCUSSION

Synthesis of azido-phospholipids

The complete reaction sequence for the synthesis of azido-phospholipids is presented in Figs. 1 and 3. In order to synthesize photoactive PE analogs, we used a heterobifunctional photoactivable compound, HSAB, coupled to the polar head group of PE to form AB-PE. The photoactive aryl azide group is about 8 Å distant from the carbonyl group as shown in **Fig. 1.** When the synthesized AB-PE was analyzed by thin-layer chromatography, a single UV spot and a major iodine-positive spot with an R_f of 0.84 was evident. Traces of UV-absorbing contaminant were also detected at the solvent front. The purified AB-PE was rechromatographed and found to be pure. The product comigrated with DNP-PE and was phosphate-positive and ninhydrin-negative. The product was obtained in high yield (78%). AB-LPE was prepared from AB-PE by phospholipase A_2 treatment and the synthesized product had an R_f of 0.38. The optical absorption spectra of AB-PE following the photoreactivity of the aryl azide is shown in **Fig. 2.** Substantial photosensitivity was exhibited by this compound as illustrated by the decrease in the absorption spectra with increasing time of UV exposure. The AB-PE absorption at 272 nm disappeared upon irradiation generating a new absorption maximum at 268 nm with lower absorption. The observed nearly isosbestic points indicated a homogeneous reaction product, thus confirming the purity suggested by other methods.

Fig. 3 shows the preparation of PC-I in which the photoactive group is on the fatty acyl chain of phospholipid. The synthesized PC-I was converted to PE by transphosphatidylation by phospholipase D in the presence of ethanolamine. The product comigrated with PE (R_f = 0.41) and was phosphate-and ninhydrin-positive. In order to demonstrate the light-sensitive activation of azido group of the synthesized lipid, an aliquot was applied on a silica thin-layer plate, UV-irradiated for 15 min at a distance of 4 cm, followed by chromatography in a solvent system described under Materials and Methods. The extent of photoactive material that remained at the origin was determined by phosphorus analysis. Irradiated samples showed that about 67% of the phosphorus-containing compound remained at the origin, suggesting that the synthesized PC-I and PE-I contain a photoactive group.

Hydrolysis of azido-phospholipids with phospholipases

The purified azido-phospholipids were subjected to hydrolysis by phospholipases (**Table 1**) as described in detail by Kates (18). Lipase and phospholipase A_2 efficiently utilized N-acylated PE and azido acyl PE as substrates. Phospholipase C and D hydrolyzed PC-I and PE-I to give respective diacylglycerides and phosphatidic acids. On the other hand, AB-PE was a very poor substrate for phospholipase C and D, yielding less than 1% of enzyme activities observed with PE, PC-I, and PE-I as substrate under the same conditions. This lack of hydrolysis was probably due to the steric hindrance imposed by the aromatic moiety present in the AB-PE. Similar observations had also been reported earlier for the *N*-[3-(3-^{125}I)iodo-4-hydroxybenzyl)-propionylphosphatidylethanolamine (21).

Photoinactivation of phospholipase A_2 by azido-phospholipids

The extent of inactivation of phospholipase A_2 by AB-PE and PE-I was determined upon irradiation with UV light for various lengths of time (**Fig. 4**). Maximal inactivation of enzyme by PE-I was at 6 min, which represents an inhibition of 87%. On the other hand, AB-PE showed the same extent of inactivation after 9 min of irradiation.

TABLE 1. Hydrolysis of azido-phospholipids by phospholipases

Substrates	Activity (%)			
		Phospholipase		
	Lipase	A_2	C	D
PE	100 ± 7.85	100 ± 8.31	100 ± 11.52	100 ± 10.93
AB-PE	94 ± 9.84	88 ± 6.23	0.7 ± 0.11	0.4 ± 0.07
PC-I	97 ± 8.21	85 ± 7.21	91 ± 10.35	96 ± 11.32
PE-I	93 ± 5.47	90 ± 6.56	87 ± 9.64	98 ± 10.45

Lipase and phospholipase A_2 activities were quantified by estimating the released fatty acids using an enzyme-coupled colorimetric method (19). Activities of phospholipase C and D were measured by quantifying the unreacted substrate (20). Enzyme assays were carried out in the dark. Values are expressed as mean percent ± SD of five determinations.

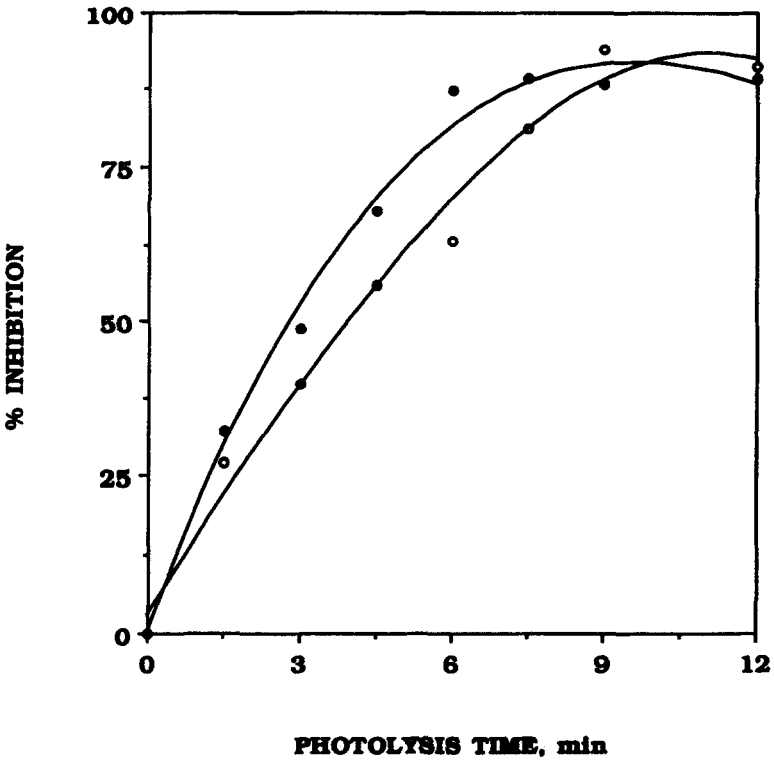

Fig. 4. Time course of photoinactivation of phospholipase A_2 by AB-PE and PE-I. The enzyme was irradiated for various lengths of time with UV and assayed for the release of fatty acids as described under Materials and Methods. Values are expressed as mean percent of three determinations. Variations among individual experiments were <11%. (○) AB-PE inhibition; (●) PE-I inhibition.

The ability of azido-phospholipids to inhibit phospholipase A_2 activity by cross-linking upon irradiation was studied under different conditions (**Table 2**). The enzyme activity was slightly affected by UV irradiation. In the presence of 0.1 mM AB-PE and PE-I, 88 and 83% of the enzyme activity was lost after 9 min of UV irradiation, respectively. When the enzyme was photolyzed in the presence of a 10-fold higher concentration of PE in addition to the azido-phospholipids, only 17–21% of the enzyme activity was lost. These results suggest that the synthesized lipid analogs were photoinserted into the substrate binding site or at the interfacial recognition site of the enzyme. It has previously been shown that *p*-bromophenylacyl bromide inactivates phospholipase A_2 by modifying a histidine residue (22). It is possible that the nitrene generated from photoactive phospholipids with aromatic or aliphatic azide could interact like *p*-bromophenylacyl bromide and thereby inactivate the enzyme. Unlike *p*-bromophenylacyl bromide, azido-phospholipids could interact either with the *N*-terminal region of the enzyme, altering the interfacial recognition site, the substrate binding site, or possibly both. The exact site of inactivation between azido-phospholipids and phospholipase A_2 remains to be established.

TABLE 2. Photoinactivation of phospholipase A_2 by PE analogs

Additions	UV Irradiation	Activity (%)
0.1 mM PE	–	100 ± 8.84
0.1 mM PE	+	89 ± 10.33
0.1 mM AB-PE	–	97 ± 6.57
0.1 mM AB-PE	+	12 ± 0.95
0.1 mM PE-I	–	94 ± 7.02
0.1 mM PE-I	+	17 ± 1.67
0.1 mM AB-PE + 1 mM PE	+	83 ± 9.08
0.1 mM PE-I + 1 mM PE	+	79 ± 8.76

Enzyme (5 U, 0.1 ml) was photolyzed in 25 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$ and 0.1 mM sonicated dispersions of azido-phospholipid. The enzyme was assayed using PE as substrate as described under Materials and Methods. Photolysis was performed by placing the samples on ice and irradiating with an ultraviolet lamp at a distance of 8 cm for 9 min. The presence or absence of UV light prior to assay is indicated by + or –, respectively. Values are expressed as mean percent ± SD of three analyses.

We have prepared two types of PE, one that carries photoactivable aryl azide in the polar head group and another that carries the azido group on the acyl moiety of PE. Our results indicate that the azido analogs of PE will prove to be useful in identifying and characterizing the membrane-bound LPE and PE-utilizing enzymes in oilseeds.

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