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DIFFERENTIAL HYDROLYSIS OF IMMOBILIZED PHOSPHATIDYLCHOLINES BY PHOSPHOLIPASES A_2 AND C

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A novel phospholipid, 1-fatty acyl-2-(12-aminododecyl) phosphatidylcholine (APC), was synthesized and reacted with two different activated agarose matrices, differing in the spacer arm length: *N*-hydroxysuccinimidylester agarose (1-atom spacer arm) and *N*-hydroxysuccinimidylester-6-aminohexanoic acid agarose (8-atom spacer arm). Both immobilized phosphatidylcholines were readily degraded by *Bacillus cereus* phospholipase C at similar rates. By contrast, *Crotalus adamanteus* phospholipase A_2 hydrolyzed long-spacer arm phosphatidylcholine, but had less than one tenth of the activity towards the short-spacer arm one. These results are interpreted in terms of a chain length-related steric hindrance caused by the matrix, affecting phospholipase A_2 but not phospholipase C activity, supporting the view that the first involves a deeper burrowing of the substrate into the enzyme molecule.

The catalytic mechanism of phospholipases is a subject of intensive research [1-3]. In recent years, considerable progress has taken place through the study of the crystal structure of complexes of phospholipase A_2 with phospholipid analogues [4-5]. The current view assumes the occurrence of a catalytic site well buried inside the enzyme molecule. At this site, interaction between the phospholipid polar head group, a Ca^{2+} ion and a characteristic set of side-chains of aminoacid residues accounts for binding of the substrate and hydrolysis of the fatty acyl ester at the *sn*-2 position [6]. Less information is available on other phospholipases, like PC-phospholipase C. This enzyme acts on phosphodiester bonds at the polar head groups. Its activity, however, requires the presence of fatty acyl hydrophobic moieties [7].

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Abbreviations used: APC, 1-fatty acyl-2-(12-aminododecyl) phosphatidylcholine: compound **7**; [^{3}H]-APC, ([^{3}H]-methyl) APC; DCC, dicyclohexylcarbodiimide; DG, diacyl glycerol; DMAP, 4-(dimethylamino)pyridine; EDTA, ethylenediaminotetraacetic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PC1A, APC-carbamide agarose: compound **9**, with agarose- as the resin matrix; PC8A, APC-6-aminohexanoamide agarose: compound **9**, with agarose-NH(CH_2)$_5$- as the resin matrix; PE, phosphatidylethanolamine; RT, room temperature (20-25 °C); *N*-tBOC, *N*-(*tert*-butyloxy)carbonyl; THF, tetrahydrofuran; Tris-HCl, tris(hydroxymethyl) aminomethane hydrochloride.

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The applications of agarose-attached lipids to the field of signal transduction may be of considerable interest. For instance, protein kinase C, which has pivotal roles in cell regulation [8-9], binds DG molecules. In addition, PA is now emerging as another important messenger molecule [10-11]. In this context, the study of proteins that interact with DG and PA would be substantially helped if affinity matrices carrying these lipids in an immobilized form were available. To this end, hydrolyzable immobilized PCs are required. This contrasts with previous work which sought enzyme resistant immobilized lipids [12].

Herein we report the design and synthesis of two novel phospholipid matrices (**9**: PC1A and PC8A) and their use to explore substrate recognition by phospholipases A_2 and C. A main feature of this design is the distal attachment point of the phospholipid moiety to the agarose resin, i.e. at the end of the fatty acyl chain in position *sn*-2, and separated by a variable-length spacer arm from the resin matrix. The rationale behind this was to allow enough motional freedom to the substrate phospholipid molecule in order not to impair recognition by the enzymes at the critical polar head group.

MATERIALS AND METHODS

General Procedures. ^{1}H NMR spectra were recorded on a Bruker WM 250 NMR spectrometer and FTIR spectra on a Nicolet 5SX/5DX FTIR spectrometer. Column chromatography was performed on silica gel 60 (E. Merck, Darmstadt, FRG), or Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Thin layer chromatography (TLC) was routinely performed on 5.0 x 1.6 cm plates of silica gel 60 F_{254} (E. Merck). Solvent systems employed were the following: solvent 1: chloroform: methanol: water: acetic acid (65:25:4:1, v/v); solvent 2: chloroform: methanol: ammonium hydroxide (75:25:4, v/v). Spots on the TLC plates were visualized by fluorescence quenching under illumination with a 254 nm UV light source and/or staining with various reagents: ammonium molybdate: ceric sulfate (10 g of ammonium molybdate, tetrahydrate; 0.1 g of cerium (IV) sulfate in 100 mL of 10% aqueous sulfuric acid); molybdenum blue (Sigma Chemical Co., St. Louis, Missouri) and ninhydrin (0.2% acetone solution). Occasionally, charring of the plate by immersion in 15% aqueous sulfuric acid and heating (>350 °C) on a hot plate was used to detect fatty acids and derivatives. All reagents employed were of analytical grade and were purchased from Sigma or Aldrich Chemical Co. (Milwaukee, Wisconsin). Phospholipids were purchased from Avanti Polar Lipids (Birmingham, Alabama). Phospholipase C from *Bacillus cereus* was from Boehringer (Mannheim, FRG). Phospholipase A_2 from *Crotalus adamanteus* was from Sigma. Radiochemicals were purchased from Amersham (Arlington Heights, Illinois) or New England Nuclear (Boston, Massachusetts). Solvents were purchased from Baker, Pierce and Aldrich. Chloroform was refluxed from phosphorus pentoxide, distilled before use (b.p. 59-60 °C) and stored in a dark glass bottle. THF was refluxed from CaH_2 and distilled before use. All other solvents were of reagent grade quality and used without further purification.

Radioactivity measurements. TLC plates containing ^{3}H or ^{14}C products were sprayed with En^{3}Hance (NEN), then covered with preflashed Kodak XAR-5 film for autoradiography and the film was exposed at -70 °C in the dark. Direct quantitation of radioactivity present on TLC plates (10 cm long runs) was achieved by cutting 2-3 mm wide slices along the run, scraping the silica off the glass plate and counting the samples by liquid scintillation.

Synthesis and Characterization of Compounds:

12-(*N*-tBOC)amino dodecanoic acid (2). We employed a procedure similar to that described for the synthesis of *N*-tBOC-α-aminoacids [13], with some modifications. 2.5 g of 12-aminododecanoic acid (**1**, 10.9 mmoles, 95% purity, from Aldrich or Sigma) were dissolved in 100 mL of THF: aqueous NaOH (0.5 g, 12.5 mmoles) (2:1, v/v). 1.4 g (11.5 mmoles) of DMAP were then added and the mixture was cooled to 0 °C in an ice bath. The reaction was started by the dropwise addition to the stirred solution of 2.8 g (12.8 mmoles) of di-*tert*-butyl pyrocarbonate dissolved in THF. A cloudy white precipitate appeared almost immediately. After 5 min, the stirred reaction mixture was allowed to warm up to RT. After 16 h, the solvent was evaporated under reduced pressure and diethyl ether was added to the remaining aqueous phase. After acidification with concentrated HCl (until pH=1), the aqueous layer was quickly extracted 4 times with diethyl ether. The combined ether extracts were washed firstly with dilute aqueous HCl and then with water. Finally, the ether extract was dried with $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. Traces of solvent were further removed under high vacuum. The white powder that remained was essentially pure product (**2**, 2.75 g, 80 % yield). Chromatographically pure material can be obtained after gel filtration through Sephadex LH-20, employing chloroform: methanol (1:1, v/v) as the eluant, and silica gel column chromatography in chloroform: methanol (50:1, v/v). ^{1}H-NMR ($CDCl_3$, 250 MHz): δ 1.28 (m, 16H), 1.43 (s, 9H), 1.63 (m, 2H), 2.33 (t, J=7.5 Hz, 2H), 3.07 (broad m, 2H), 4.52 (broad s, 1H). TLC developed in chloroform: methanol (7:1, v/v) showed a single spot (R_f=0.5), with characteristic "tailing", similar to that of myristic acid. Spots were detected by charring. Unlike the starting material (purple spot at R_f=0.0), the product does not react with ninhydrin.

Anhydride of 12-(*N*-tBOC)amino dodecanoic acid (3). 0.2 g (0.63 mmoles) of **2** were dissolved in 3-5 mL of freshly distilled dry chloroform and the solution was cooled in an ice bath. To this stirred solution a freshly prepared solution of 72 mg (0.35 mmoles) of DCC in dry chloroform was added dropwise. The reaction flask was gassed with dry argon and capped tightly. After 5 min at 0 °C the reaction mixture was allowed to warm up to RT. The appearance of a heavy white precipitate of dicyclohexylurea (DCU) was evident after 10-15 min. After 2.5 h, the DCU precipitate was removed by filtration through glass wool, and the solution was used immediately in the coupling reaction to egg lysophosphatidylcholine **5**. TLC analysis of this solution (in chloroform: methanol, 7:1, v/v) and charring of the plate revealed a major spot that migrates close to the solvent front (**3**).

Egg lysophosphatidylcholine (5). **5** is either produced from egg PC or purchased directly (to Avanti or Sigma). This compound is obtained from PC (**4**) following the reaction conditions already described for phospholipase A_2 hydrolysis [14]. 60 mg of egg PC (**4**, Avanti or Sigma) were dissolved in a minimum volume of chloroform and 6 mL of diethyl ether (1% methanol) were then mixed with this solution. 6 mL of 50 mM aqueous Tris-HCl buffer, 40 mM $CaCl_2$, pH 8.5 were then added. The reaction was initiated by the addition of 0.2-0.6 mL of a freshly prepared suspension of crude snake venom of *Crotalus adamanteus* (Sigma V-6875, 15 mg/mL in 200 mM borates buffer, pH 8.5). The stirred emulsion was refluxed at 37 °C for 2-5 h. TLC analysis (in solvent 1, and detection of spots by molybdenum blue staining and charring) revealed complete disappearance of the starting material and appearance of **5** (lyso PC) and free fatty acid. At the end, the ethereal phase was evaporated under reduced pressure and the remaining aqueous phase was lyophilized. Products were then extracted from the solid residue with chloroform: methanol (1:1, v/v) and separated by silica gel column chromatography. The identity of **5** was ascertained by comparison with a commercial sample of egg lyso PC.

1-fatty acyl-2-(12-(*N*-tBOC)amino dodecyl) phosphatidyl choline (6). The coupling protocol followed here was similar to that described by Gupta et al. [8-9], with modifications. 30 mg of egg lyso PC (**5**, approx. 0.066 mmoles) were dissolved in dry chloroform and the solvent was then evaporated under reduced pressure to leave a thin film on the walls of a round bottom flask. Azeotropic removal of traces of water and volatile alcohols in the sample was achieved by washing the film with dry toluene and evaporating the solvent under high vacuum (3 times). The sample was then redissolved in a minimum volume of dry chloroform and 10 mg (0.081 mmoles) of DMAP dissolved in the same solvent were added. At this point, the solution containing the previously prepared anhydride (0.315 mmoles, approx. 5 fold excess over **5**) was added dropwise with constant stirring. Care should be taken to maintain the reaction volume to a minimum, in order to maximize the yield of the reaction. The reaction vessel was finally gassed with dry argon and capped tightly. The progress of the reaction was followed by TLC (in solvent 1) and staining with molybdenum blue. After 12-14 h at RT, the reaction was essentially complete as revealed by (a) the disappearance of starting material **5** (spot with R_f= 0.1), and (b) the appearance of product **6** as a new spot (R_f= 0.45), similar in its behavior to dimyristoyl PC. At the end, the reaction mixture was washed with dilute aqueous HCl to remove DMAP, and the chloroformic phase was dried with $MgSO_4$, filtered and evaporated under reduced pressure. Product **6** (a slightly yellowish transparent oil, >84% yield) was purified by preparative silica gel TLC (Anasil, 20 x 20 cm, 2 mm thickness, developed in solvent 1). Alternatively, silica gel column chromatography, employing firstly a mixture of chloroform: methanol: water: acetic acid (88:26:4:1, v/v) and then solvent 1, can be used. For analytical purposes, a final filtration is carried out through a Sephadex LH-20 column eluted with chloroform: methanol (1:1, v/v). Following this same protocol, radioactively labeled **6** was obtained from **5** (containing either [^{14}C] or [^{3}H] in the choline moiety). ^{1}H-NMR ($CDCl_3$, 250 MHz): The spectrum of **6** showed characteristic broadening typical of phospholipids, δ 0.86 (t, J=6.3 Hz, 3H), 1.27 (m, 36-40H), 1.43 (s, 9H), 1.58 (m, 6H), 2.28 (overlapping triplets, 4H), 3.07 (m, 2H), 3.28 (s, 9H), 3.77 (broad, 2H), 3.94 (t, 2H), 4.11 (dd, 1H), 4.33 (broad and complex, 2H), 4.52 (broad, 1H), 5.17 (m, 1H). TLC analysis of product **6** revealed a single spot after staining with molybdenum blue or charring that did not stain with ninhydrin (Rf= 0.45, in solvent 1; Rf= 0.25, in solvent 2).

1-fatty acyl-2-(12-amino dodecyl) phosphatidylcholine (7, APC). Reaction conditions were similar to those described before [14], except that chloroform was used as the solvent instead of dichloromethane. Compound **6** (10-30 mg) was dissolved in 1-2 mL of chloroform and cooled to 0 °C in an ice bath. To this stirred solution, a few drops of methanol and an equal volume of ice-cold trifluoroacetic acid (TFA) were added dropwise. The flask was flushed with argon and capped tightly. After 30-45 min at 0 °C, the solvent was eliminated by evaporation under high vacuum at 0 °C. Traces of TFA were eliminated by repeated washings with a chloroform: methanol (1:1, v/v) solution and evaporation under high vacuum. The pale-yellowish transparent oil that remained was essentially pure **7** (80-85% yield). Further purification of this product was achieved by silica gel column chromatography and gel-filtration through a Sephadex LH-20 column eluted with chloroform: methanol (1:1, v/v). ^{1}H-NMR ($CDCl_3$, 250 MHz): The spectrum of **7** showed characteristic broadening typical of phospholipids, δ 0.86 (t, J=6.3 Hz, 3H), 1.27 (m, 36-40H), 1.58 (m, 6H), 2.25 and 2.29 (overlapping triplets, 4H), 2.82 (m, 2H), 3.27 (s, 9H), 3.73 (broad m, 2H), 3.90 (broad m, 2H), 4.01-4.44 (complex m, 4H), 5.18 (m, 1H). FTIR (neat, film): wavenumber cm^{-1} 3430 (broad, w), 2920 (s), 2850 (s), 1738 (s), 1689 (m), 1464 (w), 1363 (w), 1240 (m), 1201 (m), 1175 (m), 1126 (m), 1089 (m), 1065 (m), 969 (w), 826 (w), 800 (w), 719 (w), 576 (w). TLC analysis of product **7** revealed a single spot after staining with molybdenum blue or charring that stained purple with ninhydrin (Rf= 0.25, in solvent 1; Rf≤ 0.10, in solvent 2).

Immobilized phosphatidylcholines in agarose (PC1A and PC8A, 9). Two activated resins **8** differing in the length of the spacer arm, namely, *N*-hydroxysuccinimidylester agarose (Sigma H-8635, 1-atom spacer arm) and *N*-hydroxysuccinimidylester-6-aminohexanoic acid-agarose (Sigma A-9019, 8-atom spacer arm) were coupled to APC (**7**) to yield the two immobilized PCs (**9**), namely, PC1A and PC8A, respectively. APC (**7**) was mixed with radiolabeled APC (with [^{3}H]-methyl groups in the choline moiety) to a specific activity of $1.25 \cdot 10^5$ cpm/mmole and dissolved in THF: 100 mM aqueous $NaHCO_3$, pH 8.0, (3:1, v/v). The gels were washed, resuspended in the same solvent and mixed with the solution of APC at a ratio of 40 mmoles of phospholipid/mL of settled gel. After shaking the suspensions for 15 h at RT, the gels were washed 3 times with the solvent and 3 times with chloroform: methanol (1:1, v/v). Any unreacted carboxylate groups were blocked by incubation with 1.5 M Tris-HCl, pH 8.0 for 1.5 h. After a final wash with water, radioactivity in samples from the settled gels was measured by liquid scintillation counting. Under these conditions, 5 or 12 mmoles of APC covalently couple to each mL of 1- or 8-atom spacer arm resins, respectively, to yield **9** (PC1A or PC8A). After 60 days at 4 °C, less than 1% of the radioactivity originally associated with the gels was spontaneously released to the aqueous medium.

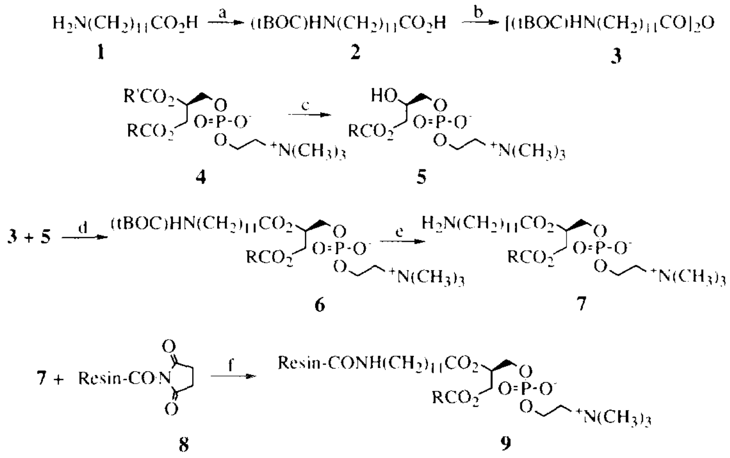
Assay of phospholipase activities on immobilized phosphatidylcholines.

Phospholipase A_2. The reaction mixture contained 1.1 mL of 50 mM Tris-HCl, pH 8.1, 1 mM EDTA, 5 mM $CaCl_2$, 0.1 mL of a suspension of immobilized PC in water, (0.2 mmoles/mL of phospholipid in the final incubation mixture), and 3 mL of phospholipase A_2 (from *Crotalus adamanteus*, 4 mg/mL) or buffer for the blanks. This mixture was incubated at RT with shaking by inversion. At the indicated time points, two 50 mL aliquots of the incubation mixture were removed and diluted to 0.5 mL with 50 mM Tris-HCl, pH 8.1, 10 mM EDTA. After brief centrifugation, the radioactivity in 0.4 mL of the supernatant was measured by liquid scintillation counting.

Phospholipase C. The activity of phospholipase C (from *Bacillus cereus*, 4000 U/mL) was assayed similarly to phospholipase A_2, in 30 mM sodium borate, 50 mM Tris-HCl buffer (pH 8.1).

RESULTS AND DISCUSSION

Synthesis of immobilized PCs: The synthesis of immobilized PCs in agarose is outlined in Figure 1. Briefly, the amino group in 12-aminododecanoic acid **1** is masked as the *N* -tBOC derivative **2** before the carboxylate is activated as the homologous anhydride **3**. Egg PC **4** is digested with phospholipase A_2 (from *Crotalus adamanteus* venom) to yield lyso-PC **5**. The key step in this synthesis is the efficient coupling procedure of **5** to **3** in the presence of DMAP to yield 1-fatty acyl-2-(12-(*N*-tBOC) aminododecyl) phosphatidylcholine **6**. Finally, deprotection of the



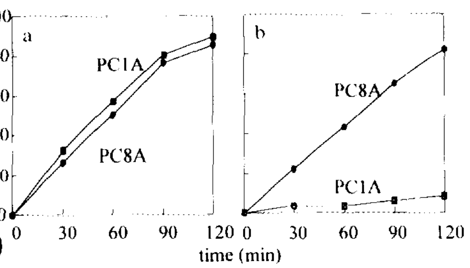
Resin-: Agarose- (9 = PC1A), Agarose-NH(CH$_2$)$_5$- (9 = PC8A)

Figure 1. Synthesis of immobilized phosphatidylcholines **9**. Reaction conditions were as follows: (a) di-*tert*-butyl pyrocarbonate and DMAP in THF: aqueous NaOH at 0 °C and later at RT, 16 h; (b) DCC in anhydrous chloroform at 0 °C and later at RT, 2.5 h; (c) *Crotalus adamanteus* venom (source of phospholipase A$_2$) suspended in 50 mM Tris-HCl buffer, 40 mM CaCl$_2$, pH 8.5, and diethyl ether (methanol 1%) at 37 °C, 2-5 h; (d) DMAP in anhydrous chloroform at 0 °C and later at RT, 16 h; (e) trifluoroacetic acid 50% in chloroform with drops of methanol at 0 °C, 30-45 min; (f) activated resins **8** suspended in THF: aqueous NaHCO$_3$, pH 8.0 at RT, 15 h.

amino function with trifluoroacetic acid yields 1-fatty acyl-2-(12-aminododecyl) phosphatidylcholine **7** (APC). Two activated resins **8** differing in the length of the spacer arm, namely, *N*-hydroxysuccinimidylester agarose (1-atom spacer arm) and *N*-hydroxysuccinimidylester-6-aminohexanoic acid-agarose (8-atom spacer arm) react readily with **7** to yield resins **9**, PC1A and PC8A, respectively.

Enzymatic activity of phospholipases on immobilized PCs: The experiments presented here show striking differences between the activities of phospholipase A$_2$ and phospholipase C towards PC1A and PC8A (Figure 2). Phospholipase A$_2$ (from *Crotalus adamanteus*) readily hydrolyzes PC8A, but shows about 10 times less activity against PC1A. By contrast, phospholipase C (from *Bacillus cereus*) has similar activities towards both substrates. We interpret these results in terms of fatty acyl chain length-related steric hindrance, affecting phospholipase A$_2$, but not phospholipase C activity, supporting the view that recognition of the substrate by phospholipase A$_2$ involves deeper burrowing of the substrate into the enzyme molecule.

Previous studies, using synthetic PCs, have revealed chain length requirements for both phospholipases. Thus, A$_2$ requires a chain length of at least 7 carbon atoms at position *sn*-2 for optimal activity [2], while the activity of phospholipase C from *Bacillus cereus* is low against dibutiroyl PC, but high against dihexanoyl PC when both substrates are assayed below the critical micellar concentration (CMC) [7]. This suggests that at least a 6-carbon long chain is required for optimal binding and hydrolysis by phospholipase C. PC1A appears to satisfy the chain length requirement for optimal activity of phospholipase A$_2$, and yet, it is only very poorly hydrolyzed by this enzyme. Our findings agree well with a recent study [17] using phosphatidylcholines in which one or both fatty acids were octanoates containing a terminal thiol group. When these substrates



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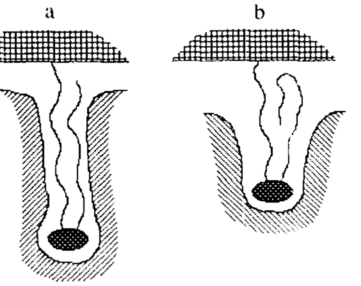

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Figure 2. (a) Phospholipase C and (b) phospholipase A_2 hydrolysis of PC1A (squares) and PC8A (circles). Phospholipase C (*Bacillus cereus*) was assayed at RT in 30 mM sodium borate: 50 mM Tris-HCl, pH 8.1 with a shaken suspension of immobilized [^{3}H]-APC (PC1A or PC8A) in water (equivalent to 0.2 mM final phospholipid concentration). Phospholipase A_2 (*Crotalus adamanteus*) was assayed in a similar fashion, except that the buffer was 50 mM Tris-HCl, pH 8.1, 1 mM EDTA, 5 mM $CaCl_2$. At each indicated time point, samples from the incubation mixture were removed and diluted with 50 mM Tris-HCl, pH 8.1, 10 mM EDTA. Radioactivity in the supernatant was measured by liquid scintillation counting.

Figure 3. Schematic illustration of the productive interaction of immobilized PCs within the active sites of phospholipases: (a) Phospholipase A_2 bound to PC8A and (b) Phospholipase C bound to PC1A. Increased steric hindrance by the resin matrix, restraining the vertical span of the substrate in (b) may account for the lower activity of phospholipase A_2 towards this substrate.

were in the monomeric form, both enzymes were active, but upon dimerization or polymerization, they became poor substrates for phospholipase A_2 but not for C, i.e. alterations in distal portions of the hydrophobic chain have dramatic effects on phospholipase A_2 without affecting C activity. Results with these two different types of tethered substrates point out that not only chain length is relevant, but also restraints imposed on the substrate molecule by bulky groups anchoring the terminal portion of the fatty acyl chain will strongly affect phospholipase A_2 activity. Figure 3 illustrates these concepts schematically.

An additional factor that could contribute to explain our results is the following. As immobilized PCs are not expected to aggregate into micellar or bilayer structures, the hydrophobic fatty acyl chains are forced to interact with the aqueous medium. Under these conditions, it is likely that the two fatty acyl chains maximize contact with each other in order to minimize the surface exposed to water. While this effect can be attained by a simple parallel arrangement of the chains in the case of PC8A, this is not possible in the case of the short spacer arm since the fatty acyl chain at the *sn* -1 position is 16-18 atoms long, whereas the fatty acid covalently linked to agarose at the *sn* - 2 position is only 12. Because of this difference in length, the *sn* -1 fatty acid may fold as shown schematically in Figure 3. The presence of "triple-chain" structures might produce an additional steric hindrance effect.

Immobilized PCs described here may potentially serve as a source of new affinity chromatography media for PC binding proteins as well as for DG and PA ligands. Our observations on the importance of spacer arm length may be used to confer selectivity to these affinity media towards the chosen protein ligands. On the other hand, the versatility of the synthetic procedure and the fact that these immobilized PCs are susceptible to enzymatic modification allows one to change the nature of the phospholipid head group and, thus, obtain affinity media bearing other specific phospholipids or neutral lipids (e.g. phosphatidylinositol, phosphatidylethanolamine, di and monoacyl glycerol instead of PC) without compromising the general synthetic scheme for the attachment to the agarose matrix.

Finally, the fact that immobilized diacyl phospholipids are attacked by different phospholipases suggests that these novel materials can also be employed for simplified assays of these activities. The main advantages of the use of immobilized phospholipids as enzyme substrates are: (i) they eliminate the need for manipulation of lipids dissolved in organic solvents, as they are directly available in an aqueous suspension, and (ii) separation of the products from the substrate is easily attained by centrifugation or filtration. Neither extraction nor TLC are thus required. The released products can then be measured by chemical methods or, more conveniently, by scintillation counting, in the case of radiolabeled immobilized lipids. We have observed that the addition of 1% Triton X-100 to the reaction mixtures did not affect the activities of either phospholipase A_2 or C towards PC8A (results not shown). On one hand, this result dramatically substantiates the notion that, when added to phospholipid suspensions, the detergent affects the substrate and not the enzyme molecule. On the other hand, this observation indicates that the assay using immobilized PCs is largely independent from the surfactants present in the medium, a factor difficult to evaluate when complex samples are tested and that can severely affect the results obtained using conventional phospholipid substrates.

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REFERENCES

1. Several articles on phospholipases edited by Dennis, E.A. (1991) *Methods Enzymol.* **197**.
2. Roberts, M.F. (1991) *Methods Enzymol.* **197**, 95-112.
3. Barlow, P.N., Lister, M.D., Sigler, P.B., and Dennis, E.A. (1988) *J. Biol. Chem.* **263**, 12954-12958.
4. White, S.P., Scott, D.L., Otwinowski, Z., Gelb, M.H., and Sigler, P.B. (1990) *Science* **250**, 1560-1563.
5. Scott, D.L., Otwinowski, Z., Gelb, M.H., and Sigler, P.B. (1990) *Science* **250**, 1563-1566.
6. Scott, D.L., White, S.P., Otwinowski, Z., Yuan, W., Gelb, M.H., and Sigler, P.B. (1990) *Science* **250**, 1541-1546.
7. El-Sayed, M.Y., DeBose, C.D., Coury, L.A., and Roberts, M.F. (1985) *Biochim. Biophys. Acta* **837**, 325-335.
8. Nishizuka, Y. (1984) *Nature (London)* **308**, 693-698.
9. Nishizuka, Y. (1986) *Science* **233**, 305-312.
10. Billah, M.M., and Anthes, J.C. (1990) *Biochem. J.* **269**, 281-291.
11. Exton, J.H. (1990) *J. Biol. Chem.* **265**, 1-4.
12. Rock, C.O., and Snyder, F. (1975) *J. Biol. Chem.* **250**, 6564-6566.
13. Moroder, L., Hallett, A., Wünsch, E., Keller, O., and Wersin, G. (1976) *Hoppe Seyler's Z. Physiol. Chem.* **357**, 1651-1653.
14. Delfino, J.M., Schreiber, S.L., and Richards, F.M. (1993) *J. Am. Chem. Soc.* **115**, 3458-3474.
15. Gupta, C.M., Radhakrishnan, R., and Khorana, H.G. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4315-4319.
16. For the synthesis of modified fatty acids and glycerophospholipid analogs, see also: Radhakrishnan, R., Robson, R.J., Takagaki, Y., and Khorana, H.G. (1981) *Methods Enzymol.* **72**, 408-433 and addendum 793-794, and references cited therein.
17. Soltys, C.E., Bian, J., and Roberts, M.F. (1993) *Biochemistry* **32**, 9545-9552.