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Factors regulating the substrate specificity of cytosolic phospholipase A_2-alpha in vitro

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ABSTRACT

Cytosolic phospholipase A_2 alpha (cPLA$_2\alpha$) plays a key role in signaling in mammalian cells by releasing arachidonic acid (AA) from glycerophospholipids (GPLs) but the factors determining the specificity of cPLA$_2\alpha$ for AA-containing GPLs are not well understood. Accordingly, we investigated those factors by determining the activity of human cPLA$_2\alpha$ towards a multitude of GPL species present in micelles or bilayers. Studies on isomeric PC sets containing a saturated acyl chain of 6 to 24 carbons in the *sn*1 or *sn*2 position in micelles showed an abrupt decrease in hydrolysis when the length of the *sn*1 or *sn*2 chain exceeded 17 carbons suggesting that the acyl binding cavity on the enzyme is of the corresponding length. Notably, the saturated isomer pairs were hydrolyzed identically in micelles as well as in bilayers suggesting promiscuous binding of acyl chains to the active site of cPLA$_2\alpha$. Such promiscuous binding would explain the previous finding that cPLA$_2\alpha$ has both PLA$_1$ and PLA$_2$ activities. Interestingly, increasing the length of either the *sn*1 or *sn*2 acyl chain inhibited the hydrolysis in bilayers far more than that in micelles suggesting that with micelles (loosely packed) substrate accommodation at the active site of cPLA$_2\alpha$ is rate-limiting, while with bilayers (tightly packed) upward movement of the substrate from the bilayer (efflux) is the rate-limiting step. With the AA-containing PCs, the length of the saturated acyl chain also had a much stronger effect on hydrolysis in bilayers *vs.* micelles in agreement with this model. In contrast to saturated PCs, a marked isomer preference was observed for AA-containing PCs both in micelles and bilayers. In conclusion, these data significantly help to understand the mode of action and specificity of cPLA$_2\alpha$.

1. Introduction

Arachidonic acid (AA) is a precursor of prostaglandins and other eicosanoids and thus plays a key role in signaling in mammals, particularly during inflammation [1,2]. AA can be released from various glycerophospholipids (GPLs) by a number of A-type phospholipases (PLAs), including secretory PLAs (sPLA$_2$s), cytosolic Ca^{2+}-dependent PLA$_2$s (cPLA$_2$s) and Ca^{2+}-independent PLA$_2$s (iPLA$_2$s) [3]. However, amongst those enzymes only cPLA$_2\alpha$ (a.k.a. Group IVA PLA$_2$) has a pronounced selectivity towards arachidonate-containing GPLs [4,5]. cPLA$_2\alpha$ is ubiquitously expressed in mammalian tissues and its knockout in mouse greatly reduces the release of AA from GPLs [6,7] thus demonstrating that this protein plays a central role in the production of AA-derived signaling molecules in mouse and probably in the other mammals as well. The crystal structure shows that cPLA$_2\alpha$ consists of two main domains [8]. The N-terminal C2 domain that facilitates the initial association of the enzyme with a membrane in response to increasing intracellular Ca^{2+}, which in turn induces phosphorylation of certain serine residues [9,10]. The second, catalytic domain contains a "lid" that has to move aside for a GPL molecule to enter the active site cavity of cPLA$_2\alpha$ [11].

Due to the pivotal role of cPLA$_2\alpha$ in AA-release, its substrate specificity and regulation has been studied by several groups (e.g., [12–16]). Notably, cPLA$_2\alpha$ is not fully specific for AA-containing GPLs since it also hydrolyses other polyunsaturated GPLs (including those containing a 20:5 residue in the *sn*2 position) as well as other unsaturated species, albeit less efficiently [17]. The enzyme cleaves AA from GPLs containing a *sn*1 ether or ester bond with a similar efficiency, and has also been considered unselective towards the GPL's head group structure [18].

Abbreviations: AA, arachidonic acid; GPL, glycerophospholipid; SUV, small unilamellar vesicles; LPC, lysophosphatidylcholine; cPLA$_2$, Calcium^{2+}-dependent phospholipase A$_2$; PA, phosphatidic acid; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DMPM, dimyristoyl-phosphatidylmethanol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; 16:0/C$_n$-PC, *sn*1–16:0/*sn*2-C$_n$-phosphatidylcholine (n = 6–24); C$_n$/16:0-PC, *sn*1-C$_n$/*sn*2–16:0-phosphatidyl-choline (n = 6–24); 20:4/C$_n$-PC, *sn*1–20:4/*sn*2-C$_n$-phosphatidylcholine (n = 6–24); C$_n$/20:4-PC, *sn*1-C$_n$/*sn*2–20:4-phosphatidylcholine (n = 6–24).

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Yet, cPLA$_2\alpha$ acts as a PLA$_1$, transacylase or lyso-PLA [19–21]. Regarding its lyso-PLA activity, it is intriguing that *sn*1-palmitoyl lyso-PC was hydrolyzed faster than *sn*2-palmitoyl lyso-PC *in vitro* [19]. Such regiospecificity is unexpected, considering that the enzyme is thought to mainly cleave the *sn*2 chain of intact GPLs, implying that binding of the substrate acyl chain to the active site may be promiscuous or that there is more than one lipid binding site on cPLA$_2\alpha$ [4,5].

Recently, Mouchlis and coworkers employed computer-aided modeling guided by previous deuterium-exchange mass spectrometry studies to investigate the interactions of PAPC with cPLA$_2\alpha$ [12]. Their data indicated that both the acyl chains of PAPC are fully accommodated inside a hydrophobic pocket containing aromatic residues which may favorably interact with the double bonds of the AA residue. Intriguingly, both the *sn*1 and *sn*2 chain appeared to share a common hydrophobic cavity in the modeled protein. Another simulation study [22] found marked differences in the binding of PAPC vs. DPPC (containing two 16:0 chains) by cPLA$_2\alpha$. While the *sn*2 chain of PAPC was fully embedded inside cPLA$_2\alpha$, both acyl chains of DPPC remained partially outside the protein after the simulation. Although these modeling studies suggested that accommodation of the substrate acyl chains in the active site determines the substrate specificity of cPLA$_2\alpha$, they did not provide information on the role of other factors, particularly that of substrate efflux from the bilayer [23,24], which is probably the rate-limiting step in the hydrolysis of GPLs by several other soluble PLAs [25,26]. Accordingly, we employed a high-throughput mass-spectrometric assay to study to what extent the accommodation of the substrate in the catalytic site of cPLA$_2\alpha$ and substrate efflux contribute to its marked specificity for AA-containing GPLs. Towards this end, the hydrolysis of several systematically constructed sets of GPLs were studied in both micelles and vesicle bilayers. The results suggest that while favorable accommodation of the substrate in the active site greatly contributes to the preferential hydrolysis of AA-containing GPLs by cPLA$_2\alpha$ also the efflux of the substrate from a bilayer plays a significant role. In fact, when the substrate lacked an AA residue, efflux from a bilayer appeared to be the key rate-limiting step of hydrolysis. Promiscuous binding of the acyl chains of a GPL to the active site of cPLA$_2\alpha$ most probably explains why cPLA$_2\alpha$ displays both PLA$_1$ and PLA$_2$ activities.

2. Materials and methods

2.1. Lipids and other chemicals

L-α-1-palmitoyl-2-arachidonoyl-[1-^{14}C]-PC (58 μCi/μmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA), unlabeled GPLs, lyso-PCs and sphingomyelin (SM) were obtained from Avanti Polar Lipids (Alabaster, AL) and D_3-methyl iodide from Cambridge Isotope Laboratories (Andover, MA). Ethanolamine, phospholipase D (Streptomyces species), Triton X-100 and EDTA-free protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). Silica gel 60 thin-layer plates and HPLC-grade solvents were obtained from Merck Millipore (Munich, Germany), Strep-Tactin superflow resin and desthiobiotin from IBA-Life Sciences (Göttingen, Germany), bovine serum albumin standard and Coomassie brilliant blue R-250 solution from Bio-Rad (Sundyberg, Sweden). Penicillin, streptomycin and fetal calf serum (FCS) were from Gibco Life Technologies (Bleiswijk, The Netherlands). The primary (sc-454) and secondary (Streptactin HRP-conjugate) antibodies were purchased from Santa Cruz Biotechnology and BioRad respectively. The *sn*1–16:0/*sn*2-C$_n$-PC and *sn*1-C$_n$/*sn*2–16:0-PC species were synthesized by acylating *sn*1–16:0-LPC with the anhydride of a C$_n$-fatty acid or *sn*1-C$_n$-LPC with the anhydride of 16:0-fatty acid, respectively [27]. The *sn*1–20:4/*sn*2-C$_n$-PC and *sn*1-C$_n$/*sn*2–20:4-PC species were synthesized analogously by acylating *sn*1–20:4-LPC with the anhydride of a C$_n$-fatty acid or *sn*1-C$_n$-LPC with the anhydride of 20:4-fatty acid, respectively. The D_9-labeled PC species were synthesized from the corresponding phosphatidylethanolamines by methylation with D_3-methyl iodide [28]. The purity of GPLs was confirmed by TLC and mass spectrometry and their concentrations were determined by using a phosphate assay [29].

2.2. Cloning, expression and purification of human cPLA$_2\alpha$

Full length cPLA$_2\alpha$ cDNA from the clone MGC:126350 IMAGE:40034995 (ImaGenes, GenBank™ BC114340.2) was amplified by PCR using the forward primer 5′-GTCGGTCCGCCA-CCATGTCATTTA TAGATCCTTACCAGCACA-3′ containing a *Rsr*II cleavage site and the reverse primer 5′-CTGGTACCTTAGTGGTGGTGGTG-ATGGTGATGATGAT GATGTTCGAAACGACC-TTCGATTGCTTTGGGTTTACTTAGAAACTCCTTG-3′ containing the *Bst*BI and *Kpn*I cleavage sites. The *Rsr*II- and *Kpn*I-digested PCR product was ligated into pFastBac1-vector (pFB1, Gibco Life Technologies) resulting in pFB1-cPLA$_2\alpha$-His$_{10}$ vector. Replacing the *Bst*BI-*Kpn*I fragment of pFB1-cPLA$_2\alpha$-His$_{10}$ by the annealed oligonucleotides 5′-CGAATGGAGCCACCCGCAGTTCGAG-AAAGGAGGAGGAAG CGGAGGAGGAAGCGGAGGAGGAAGCT-GGAGCCACCCGCAGTT-TGAAAA ATAGGTAC-3′ and 5′-CTA-TTTTTCAA-ACT-GCGGGTGGCTCCAGCTTCCT CCT-CCGCTTCCTCCTCCGCTTCCTCTCCTTTTCGAACTGCG-GGTGGCTCCA TT-3′ produced the pFB1-cPLA$_2\alpha$-StrepIII vector. The inserted sequences and reading frames of the constructs were verified by sequencing. The Bac-to-Bac baculoviral expression system (Gibco) was utilized for bacmid preparation and FuGENE®6 (Promega)-mediated transfection of Sf9 cells was employed to produce the recombinant baculovirus. To express the cPLA$_2\alpha$-StrepIII protein, Sf9 cell suspension (500 mL, ~10^6 cells/mL) was infected with the recombinant baculovirus at the multiplicity of 1. After incubation at 26 °C for 72 h, the cells were washed with ice-cold PBS and resuspended in 50 mL of a lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% Triton X-100, 5% glycerol) containing an EDTA-free protease inhibitor cocktail. The cells were then lysed by sonication (6 × 1 min with 30 s intervals, 40% duty cycle) and centrifuged at 50,000 ×*g* for 30 min to pellet the membranes after which the purification was carried out as earlier [26].

2.3. Immunoblotting

To assess their cPLA$_2\alpha$ content, transfected Sf9 cells were pelleted, washed with ice-cold PBS, resuspended in a lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100, 1.0% deoxycholate, 5 mM EDTA) and left on ice for 30 min. The lysate was centrifuged at 800 ×*g* for 3 min at 4 °C and 20 μg of the supernatant protein was run on a 10% SDS-polyacrylamide gel. The proteins were then transferred onto a PVDF membrane (Millipore) which was treated with 5% defatted dry milk in 50 mM Tris, pH 7.4, 150 mM NaCl containing 0.1% Tween-20. The recombinant cPLA$_2\alpha$ was detected with an *anti*-cPLA$_2\alpha$ (1:2500 dilution) or a Streptactin HRP-conjugate antibody (1:10,000 dilution). After treatment with ECL Plus detection reagent kit (GE Healthcare), the blot was scanned with Starion FLA-9000 image scanner (Fujifilm) and visualized using Image Reader FLA-9000 software.

2.4. GPL mixtures studied

To determine how the structure of the acyl chains and the head group influences GPL hydrolysis by cPLA$_2\alpha$ *in vitro*, we employed a mass-spectrometric assay devised previously [30]. This high-throughput assay allows one to determine the rates of hydrolysis of multiple GPLs present together in a macrosubstrate particle, thus avoiding any bias caused by substrate-dependent variations in the physical properties of the macrosubstrate and protein binding [31,32]. Seven different mixtures of GPL species were studied (Supplementary Table 1). The first mixture ("PC-mix") consisted of 27 PC species including: (*i*) 10 saturated species, (*ii*) 3 monounsaturated species; (*iii*) 6 diunsaturated species; (*iv*) 3 species with four double bonds; (*v*) 3 species with 6 double bonds; (*vi*) one species with eight double bonds and (*vii*) one species with 12 double bonds. The second mixture (16:0/C$_n$-PCs) consisted of 17 PC species containing a 16:0 chain in the *sn*1 position and a saturated chain of 6–