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Class specific peptide inhibitors for secretory phospholipases A2

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ABSTRACT

Phospholipases A2 (PLA2) catalyze the hydrolytic cleavage of free fatty acids from the *sn*-2 OH-moiety of glycerophospholipids. These enzymes have a number of functions, from digestion to signaling and toxicity of several venoms. They have also been implicated in inflammation and are connected to diverse diseases, such as cancer, ischemia, atherosclerosis, and schizophrenia. Accordingly, there is a keen interest to develop selective inhibitors for therapeutic use. We recently proposed a novel mechanism for the control of PLA2 activity with highly active protofibrils of PLA2 existing transiently before conversion to inactive amyloid fibrils [19]. In keeping with the above mechanism several algorithms identified ^{85}KMYFNLI^{91} and ^{17}AALSYGFYG^{25} in bee venom (bv) and human lacrimal fluid (Lf) PLA2, respectively, as a regions potentially forming amyloid type aggregates. Interestingly, in keeping with the proposed role of these sequences in the control of the activity of these enzymes, preincubation of 2 nM bvPLA2 with ^{85}KMYFNLI^{91} caused complete inhibition of PLA_2 activity while the scrambled control peptide YNFLIMK had no effect. Approximately 36% attenuation of the hydrolytic activity of LfPLA2 present in human lacrimal fluid was observed in the presence of 80 nM ^{17}AALSYGFYG^{25}.

1. Introduction

Phospholipases A2 (PLA2) constitute one of the largest families of lipolytic enzymes and catalyze the hydrolysis of the ester bond at the *sn*-2 position of glycerophospholipids, releasing free fatty acids and lysophospholipids [1,2]. PLA2 serve a number of functions, from toxicity of several venoms to phospholipid metabolism, digestion, antimicrobial activity, and cellular signaling [3]. As an example polyunsaturated fatty acids (PUFA) such as arachidonic acid cleaved from the *sn*-2 position of phospholipids become metabolized to eicosanoids (prostaglandins, leukotrienes) and various other related bioactive lipids, while lysophospholipids may convert to the platelet activating factor (PAF, [1,4]).

The PLA2 superfamily currently consists of fifteen groups and different subgroups, with a number of enzymes differing in their primary sequences, structures, and catalytic mechanism [3,5,6]. Four main classes of PLA2 are recognized *viz*. secretory sPLA2, cytosolic cPLA2, Ca^{2+}-independent iPLA2, and lipoprotein associated LpPLA2 [3]. Their catalytic mechanisms and structures are conserved with a high degree of sequence homology between PLA2 from different species [3,7]. Each of the above types has been implicated in diverse inflammatory diseases, such as cancer, ischemia, atherosclerosis, and schizophrenia [8,9].

Treatment of some of the above disorders could benefit from potent and selective inhibitors of sPLA2 activity and there is a keen interest to develop more specific PLA2 inhibitors for therapeutic use [10]. Specific inhibitors of PLA2 isoforms would also be valuable to functional studies elucidating the roles of these enzymes in different cells and tissues [2]. Several PLA2 inhibitors designed to be selective for specific PLA2 isoforms have been reported. However, these compounds inhibit not only different PLA2 isoforms, but also affect other enzymes such as diacylglycerol lipase and phosphatidate phospho-hydrolase [11–14].

The honey bee (*Apis mellifera*) venom secretory PLA2 (bvPLA2) and human lacrimal fluid (LfPLA2) belong to group III and IIA, respectively [3]. The latter is considered to be proinflammatory, constitutively expressed by a number of different cells, such as platelets [15], and is found in body fluids such as seminal plasma [16], and tears [17], further implicated in inflammation, rheumatoid arthritis, and sepsis [18]. LfPLA2 is highly expressed in human tears and its concentration in the latter has been reported to reach up to 33.9–54.5 mg/ml.

Abbreviations: AMP, antimicrobial peptides; bv, bee venom; C_{28}-O-PHPM, 1-octosanyl-2-(pyren-1-yl)hexanoyl-*sn*-glycero-3-phospho-monomethyl ester; cPLA2, cytosolic PLA2; CSSP, continuum secondary structure predictor; EM, electron microscopy; FFA, free fatty acid; Hsp70, heat shock protein 70; iPLA, Ca^{2+}-independent PLA2; Lf, lacrimal fluid; LpPLA2, lipoprotein associated PLA2; LUV, large unilamellar vesicles; lysoPC, lysophosphatidylcholine; NCBI, national center for biotechnology information; PAF, platelet activating factor; PC, phosphatidylcholine; PLA2, phospholipase A2; PoxnoPC, 1-palmitoyl-2-(9′-oxononanoyl)-*sn*-glycero-3-phosphocholine; ROS, reactive oxygen species; sPLA2, secretory PLA2; PUFA, polyunsaturated fatty acids; temB, temporin B.

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We recently proposed a novel, oligomerization based mechanism for the control of PLA2 activity with highly active protofibrils of PLA2 existing transiently before subsequent conversion to inactive amyloid fibrils, this process acting as an on–off switch for enzyme activity [19]. Along these lines we also showed heat shock protein 70 (Hsp70) in the presence of ATP to activate PLA2 *in vitro* and suggested this activation to result from Hsp70 prolonging the lifetime of the high activity oligomers, counteracting inhibition due to conversion to amyloid [20]. Interestingly, PLA2 is activated by several antimicrobial peptides (AMP, [21]) and in the presence of its hydrolytic products *e.g.* lysoPC and temporin B the formation of amyloid cofibers has been observed [22,23].

In keeping with the above mechanism of control of PLA2 activity, PLA2 sequences possess structural features promoting oligomerization [19,24]. A rational approach to the design of synthetic inhibitors could be synthetic peptides interfering with PLA2–PLA2 interactions. We employed bioinformatics algorithms *viz.* CSSP [25], AGGRESCAN [26], TANGO [27], and PASTA [28] to identify in the sequences of bv and LfPLA2 regions with conformational ambiguity, as well as propensity to aggregation and amyloid formation. Subsequently, synthetic peptides corresponding to the identified regions were made and characterized for their effects on PLA2 activity *in vitro*. Phospholipid hydrolysis by sPLA2 was measured using a pyrene labeled fluorescent phospholipid analog [29] both in the absence and presence of the synthetic peptides corresponding to the amyloidogenic, aggregation promoting regions of the above two sPLA2. Our data show that these designed peptides do inhibit the catalytic activities of these enzymes, thus lending support to our proposal for functional lipid-induced oligomerization and amyloid formation as an on–off switch in the control of the catalytic activity of PLA2.

2. Materials and methods

2.1. Materials

1-Octosanyl-2-(pyren-1-yl)hexanoyl-*sn*-glycero-3-phosphatidylmonomethyl ester (C_{28}-O-PHPM) was from Invitrogen (Eugene). Its purity was verified by thin layer chromatography on silicic acid coated plates (Merck, Darmstadt, Germany) developed with a chloroform/methanol/water mixture (65:25:4, v/v/v). Examination of the plates after iodine staining or upon UV-illumination revealed no impurities. The concentration of the pyrene labeled lipid was determined from absorbance at 342 nm using 42,000 cm^{-1} as the molar extinction coefficient in ethanol. Concentrations of other lipid stock solutions in chloroform were determined gravimetrically with a high-precision electrobalance (Cahn, Cerritos, CA, or SuperG, Kibron Inc., Espoo, Finland) as described [21]. BvPLA2 was from Sigma and its purity was verified by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Synthetic peptides (KMYFNLI, YNFLIMK, and AALSYGFYG) were made by Genscript Corporation (Piscataway, NJ, USA). Freshly deionized filtered water (Milli RO/Milli Q, Millipore Inc., Jeffrey, NH) was used in all experiments. $CaCl_2$ solution was prepared and filtered through a 0.02 μM filter (Schleicher and Shuell Microscience, Dassel, Germany) prior to use. Tears were collected from healthy volunteers after a briefly exposure to the vapors from freshly minced onions. The collected lacrimal fluid was stored at −20 °C until use.

2.2. Algorithms identifying amyloidogenic regions

The primary sequences of bv and human Lf PLA2 (of group II and III PLA2, respectively, retrieved from NCBI) were analyzed by the CSSP algorithm for the identification of regions with conformational flexibility [25], as well as for susceptibility for aggregation and amyloid formation, as predicted by AGGRESCAN [26], TANGO [27], and PASTA [28].

2.3. Assay for PLA2

PLA2 activity was determined by the kinetic assay described previously [30]. Appropriate amounts of the stock solution of the pyrene-labeled PLA2 substrate C_{28}-O-PHPM [31] were dried under a stream of nitrogen, followed by high vacuum for a minimum of 2 h. The lipid residues were subsequently dissolved in ethanol to yield 50 μM lipid concentration, and five 10 μl aliquots of this ethanolic lipid solution were rapidly injected into the buffer with a Hamilton microsyringe to obtain a final concentration of 1.25 μM C_{28}-O-PHPM in a total volume of 2 ml. Fluorescence intensities were measured with a Varian Carey spectrofluorometer using magnetically stirred four window quartz cuvettes (with 10 mm path length) at 37 °C with excitation wavelength of 344 nm, and emission recorded at 400, with both emission and excitation bandpasses set at 5 nm. After 5 min equilibration the reactions were initiated by adding 4 pmol/2 ml of bv PLA2 or 1 μl of human lacrimal fluid, as indicated. The progress of phospholipid hydrolysis was followed by measuring pyrene monomer emission intensity at 400 nm as a function of time. The assay was calibrated by adding known picomolar aliquots of (pyren-1-yl)hexanoate into the reaction mixture in the absence of enzyme while recording pyrene monomer emission. The activity of the enzyme was calculated from the rate of increase in pyrene emission, converted to pmoles FFA min^{-1} ml^{-1}.

The effects of the synthetic peptides on PLA2 activity were investigated by incubating bvPLA2 or human lacrimal fluid PLA2 with the indicated peptides in buffer (5 mM Hepes, 0.1 mM EDTA, pH 7.4) at 37 °C. After the indicated incubation time, aliquots were taken from these mixtures and assayed for PLA2 activity. Alternatively, when indicated 2 or 4 nM (final concentration) of inhibitor was added into the cuvette containing 1.25 μM C_{28}-O-PHPM 5 min prior to the addition of the specified PLA2. All reactions were repeated 3–4 times and standard deviations for data points were obtained for the curves using Origin 8.1 (OriginLab Corporation, Northampton, USA).

In control experiments, a scrambled peptide (YNFLIMK) was used in which the amino acid composition was identical to that of the KMYFNLI, but the sequence was randomized.

3. Results

3.1. Rational design of possible peptide inhibitors for sPLA2

^{85}KMYFNLI^{91} and ^{17}AALSYGFYG^{25} were identified in the sequences of bv and human lfPLA2, respectively, by the AGGRESCAN [26], TANGO [27], and PASTA [28] algorithms as a regions potentially forming amyloid type aggregates. Importantly, conformational switches (residues 87–95 and 18–29, respectively) predicted in the sequence of these PLA2 by CSSP [25] coincide with the above high packing density aggregating and amyloidogenic regions (Table 1). Accordingly, these short amino acid stretches in PLA2, show propensities for random coil, α-helix and β-sheet formation, as well as for self-assembly, aggregation and oligomerization into amyloid fibrils [32] and could mediate PLA2–PLA2 interactions, resulting in the formation of functional high activity oligomers and inactive amyloid by these enzymes. As a consequence, synthetic peptides corresponding to these sequences are readily expected to interfere with the functional oligomerization of PLA2 and to act as inhibitors.

Table 1
Identification of oligomerization mediating sequence in sPLA2.

PLA2	Regions identified by				Regions identified
	CSSP[a]	TANGO[b]	PASTA[c]	AGGRESCAN[d]	
Phospholipase A2 (Apis mellifera) IIYPGTLWCGHGNKS SGPNELGRFKHTDAC CRTHDMCPDVMSAG ESKHGLTNTASHTRL SCDCDDKFYDCLKN SADTISSYFVG**KMYF** **NLI**DTKCYKLEHPVT GCGERTEGRCLHYT VDKSKPKVYQWFDL RKY	6–10, 14–34, 38–48, 55–63, 87–95, 101–110, 112–115, 117–120, 122–133	78–83, 86–91, 125–128	78–91	5–9, 78–91, 125–129	^{85}KMYFNLI^{91}
Non-pancreatic secretory phospholipase A2 NLVNFHRMIKLTTGK E**AALSYGFYG**CHCG VGGRGSPKDATDRC CVTHDCCYKRLEKR GCGTKFLSYKFSNSG SRITCAKQDSCRSQL CECDKAAATCFARN KTTYNKKYQYYSNK HCRGSTPRC	18–29, 39–45, 50–57, 62–65, 83–91, 103–111	17–25	1–9, 42–53	5–12, 19–29, 61–67	^{17}AALSYGFYG^{25}

[a] Continuum secondary structure predictor identified regions with ambivalent secondary structures.
[b] Amyloidogenic regions predicted by TANGO.
[c] Amyloidogenic regions predicted by PASTA.
[d] Aggregation-prone segments (hot spots) predicted by AGGRESCAN.

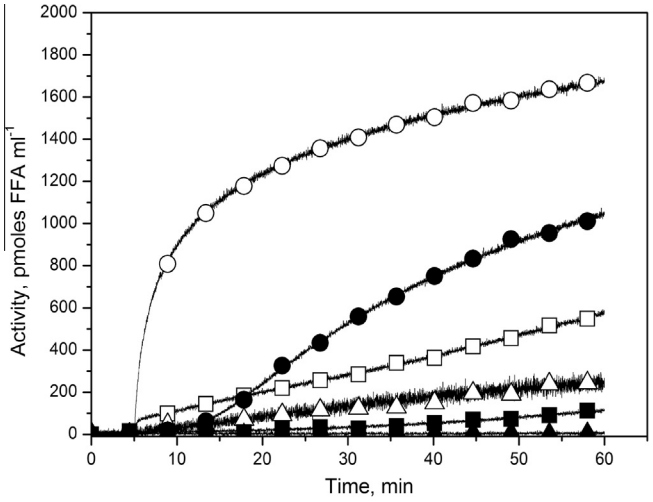

Fig. 1. Inhibition of bvPLA2 by ^{85}KMYFNLI^{91}. Hydrolysis of 1.25 μM C_{28}-O-PHPM by 2 nM bvPLA2 (○) was measured as a function of time in the presence of 2 (□), or 4 nM (Δ) peptide added to the substrate (without preincubation). Alternatively, 4 pmol of the peptide was preincubated with 4 pmol of bvPLA2 for 5 (●), 10 (■), and 20 (▲) min prior to the addition to the substrate. Reactions were carried out in 2.0 ml of 5 mM Hepes, 0.1 mM EDTA, 1 mM $CaCl_2$, pH 7.4 at 37 °C with continuous magnetic stirring.

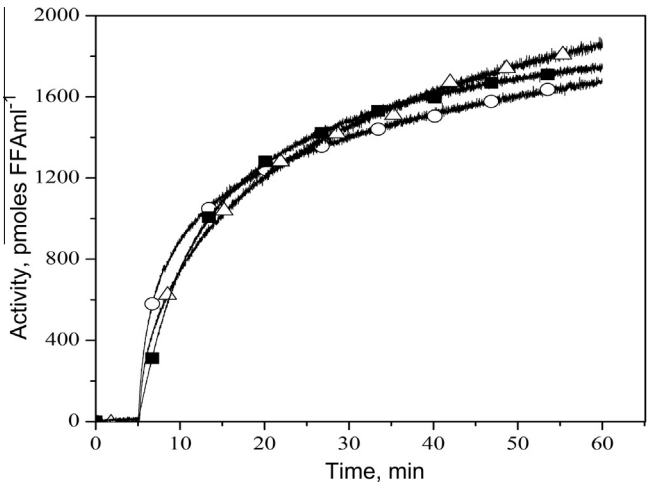

Fig. 2. Hydrolysis of 1.25 μM C_{28}-O-PHPM by 2 nM bvPLA2 (○) was measured as a function of time in the presence of 80 nM of YNFLIMK (□), and ^{17}AALSYGFYG^{25} (Δ) preincubated with the enzyme for 10 min prior to the addition into the substrate solution. Reactions were monitored in 2.0 ml of 5 mM Hepes, 0.1 mM EDTA, 1 mM $CaCl_2$, pH 7.4 at 37 °C with continuous magnetic stirring.

Hydrolysis of the negatively charged phospholipid analog C_{28}-O-PHPM by bvPLA2 starts immediately with no measurable preceeding lag [33]. Activity of bvPLA2 was decreased by 77–85% when 2 nM KMYFNLI was added to 1.25 μM C_{28}-O-PHPM 5 min prior to the addition of the enzyme (Fig. 1). More pronounced attenuation in the hydrolysis of C_{28}-O-PHPM was observed in the presence of 4 nM of KMYFNLI (Fig. 1). Further, the inhibitory effect of this synthetic peptide was also evident when, bvPLA2 was preincubated with KMYFNLI for 5, 10 and 15 min. prior to the assay of the hydrolytic reaction. Preincubation for 20 min of 2 nM PLA2 with 2 nM peptide caused complete inhibition of the PLA2 activity (Fig. 1). Importantly, control scrambled peptide YNFLIMK and AASLSYGFYG did not influence bvPLA2 (Fig. 2).

We then investigated the effects of the peptide AALSYGFYG derived from the sequence of LfPLA2 (Table 1), and studied its effects on the activity of this PLA2 *in vitro* (Fig. 3). Similarly to bvPLA2 and KMYFNLI, the peptide AALSYGFYG inhibited LfPLA2. However, the activity of LfPLA2 was decreased much less by 28% also when 40 nM peptide inhibitor was added to substrate prior to the addition of the enzyme (Fig. 3). More pronounced attenuation, approximately 36% inhibition of the hydrolysis of C_{28}-O-PHPM was observed when the concentration of AALSYGFYG was increased to 80 nM (Fig. 3). The lack of complete inhibition may reflect the presence in human lacrimal fluid of more than 1500 proteins and

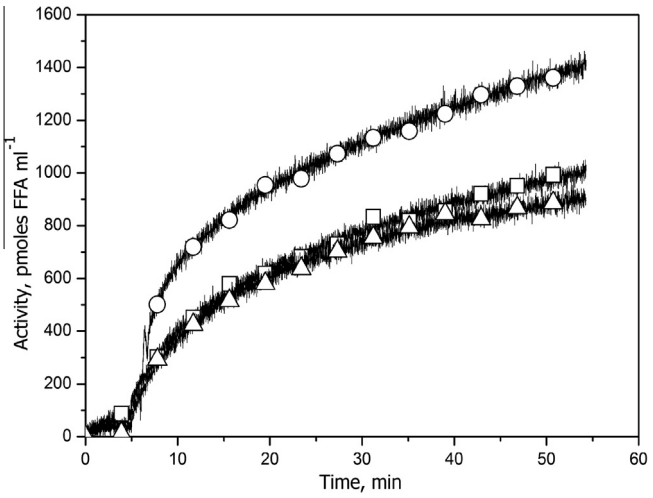

Fig. 3. Hydrolysis of 1.25 μM C_{28}-0-PHPM by 1 μl (○) LfPLA2 as a function of time in the presence of 40 (□), and 80 (Δ) nM ^{17}AALSYGFYG^{25} preincubated with the enzyme for 5 min prior to the addition into the substrate solution. Reactions were monitored in 2.0 ml of 5 mM Hepes, 0.1 mM EDTA, 1 mM $CaCl_2$, pH 7.4 at 37 °C with continuous magnetic stirring.

peptides [34], many of which have antimicrobial activity. Supporting this possibility the hydrolytic activity of LfPLA2 is significantly enhanced in the presence of AMP [21]. Moreover, several isoforms of sPLA2 have been reported in class II [11] and could also be present in the lacrimal fluid. Importantly, KMYFNLI had no effect on the activity of LfPLA2 (data not shown).

4. Discussion

The aim of the present study was to identify potential amyloid-forming regions in in the sequence of PLA2 and to study if peptides corresponding to these sequences could interfere with PLA2–PLA2 interactions, and inhibit PLA2.

Exposed specific stretches of amino acid 'hot spots' are prone to aggregate and form amyloid-like fibers [35]. Several factors including hydrophobicity, propensity towards α-helical or β-sheet structures, and net charge modulate the aggregation of such regions in a protein [36]. Such hydrophobic motifs drive proteins to form β-sheet structures, characteristic to amyloid fibers. The regions 76–92, and 17–25 in bv and LfPLA2, respectively, were identified by AGGRESCAN [26], TANGO [27], and PASTA [28] algorithms (predicting hydrophobic sequences involved in aggregation and amyloid type fiber formation) as potential hot spots for causing aggregation and amyloid formation. Importantly, conformational switches predicted by CSSP [25] in these PLA2 coincide with the above high packing density aggregating and amyloidogenic regions (Table 1).

The amyloidogenic amino acid stretch 78–92 in bvPLA2 (Table 1) belongs to the interfacial recognition site (Ca^{2+} binding loop and 76–91 helix), which binds to the substrate [5]. The above C-terminal sequence is also implicated in the neurotoxicity of bvPLA2, demonstrated in mice administered with site-directed mutants of bvPLA2 [37,38]. The activity of sPLA2 is modulated by several factors and needs to be critically controlled. Temporin B is one of the smallest AMP and has been proposed to influence the conformational equilibrium of PLA2 by forming higher order co-oligomers [22] thus activating PLA2 [21]. Along these lines the catalytic rate of PLA2 can be modulated by amyloidogenic peptides, *e.g.* amyloid Aβ-peptide [39], several AMP, *e.g.* temporin B and L, indolicidin, magainin 2 [21], LL-37 [40], bombolitin III [41], and melittin [42] which all enhance the activity of PLA2. Oxidized phospholipid derivatives bearing an aldehyde function, such as 1-palmitoyl-2-(9′-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine (PoxnoPC) can also activate PLA2 and induces the formation of thioflavin T staining fibrils [23].

Here we show that peptides derived from putative oligomerization mediating sequences can inhibit PLA2. We have suggested the interfacial activation and oligomerization of PLA2 to be interconnected processes [19]. Upon the binding of PLA2 to the substrate interface protofibrillar oligomers, PLA2 aggregates [43–45] initially dimers [46] with high catalytic activity are formed, and later convert it into inactive amyloid-like fibrils [19]. As mentioned above a shift in oligomerization can be induced by peptides that bind to the enzyme *via* non-specific and specific interactions.

The above mechanism of enzyme activity control can be expected to be more widely found in nature. Analogously, peptides derived from the HIV-1 integrase have been shown to inhibit its activity and promote the formation of inactive oligomers [47,48]. Identification of this type of structures in different enzymes could be used to obtain specific and potent peptide inhibitors. Such peptides could be made with additional cell membrane permeating sequences, allowing their entry into cells. A number of other human PLA2 were also identified with structures fulfilling the required criteria. Along these lines, we expect human PAF acetyl hydrolase as well as Hsp70 to be amongst enzymes which could eventually be targeted by this strategy.

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