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Synthetic peptide from lipocortin I has no phospholipase A_2 inhibitory activity

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Two anti-inflammatory peptides corresponding to a high amino acid similarity region between lipocortins were synthesized and tested on their ability to inhibit porcine pancreatic phospholipase A_2. Kinetic assays using monomeric and aggregated phospholipids did not reveal any phospholipase A_2 inhibitory activity. The peptides did not inhibit phospholipase A_2 activity on monolayers of negatively charged substrate and did not prevent phospholipase A_2 action on mixed micelles of 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine and sodiumdeoxycholate. Ultraviolet difference spectroscopy did not show binding of the peptides to phospholipase A_2. Therefore we conclude that these anti-inflammatory peptides do not inhibit pancreatic phospholipase A_2 in vitro, in contrast to the results recently published [(1988) Nature 335, 726–730].

Lipocortin; Phospholipase A_2; Synthetic peptide

1. INTRODUCTION

The production of the prostaglandins, leukotrienes and related eicosanoids depends on the availability of free arachidonic acid. The control of the biosynthesis of these compounds in inflammatory cells and other cells depends on the enzymatic release of arachidonic acid from the *sn*-2 position of membrane phospholipids. Phospholipase A_2 (PLA2) is the simplest and most obvious candidate for the responsible enzyme, although many types of phospholipases have been implicated [1]. Inhibition of PLA2 may provide an effective treatment of inflammatory diseases when both the cyclooxygenase and lipooxygenase pathways in the arachidonate cascade are blocked [2].

It is known that *sn*-1-lecithins are pure competitive inhibitors for PLA2: they bind with an identical affinity to the catalytic site but are not hydrolyzed [3]. Substrate analogues containing an amide or ether bond at the *sn*-2 position have been reported to be potent reversible PLA2 inhibitors [3–6].

Recent research has concentrated on the role of phospholipase A_2-inhibitory proteins as possible anti-inflammatory agents. Lipocortins are proteins that have been reported to inhibit intracellular PLA2. Their inhibitory activity towards PLA2 appeared to be regulated by phosphorylation [7,8]. Lipocortins are 36 kDa anti-inflammatory proteins which are inducible by steroids, secreted by cells, and thought to act prior to the cyclooxygenase pathway by inhibition of a phospholipase A_2. Although these proteins are thought to interact with intracellular PLA2, their inhibitory activity is usually measured with the extracellular porcine pancreatic phospholipase A_2. It has been suggested that the mechanism of lipocortin action is by direct protein–protein interaction [9–11].

Davidson et al. [12] reported that several forms of calpactin, which are lipocortin-like proteins, just as lipomodulin, macrocortin, renocortin and

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Abbreviations: PLA2, phospholipase A_2; *t*-Boc, *tert.* butyloxycarbonyl; O-Bu^t, *tert.* butylester; Cl_2-Z, dichloro-benzyloxycarbonyl; DOC, sodium deoxycholate; CMC, critical micellar concentration; HPLC, high-performance liquid chromatography

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uteroglobin [13], inhibit porcine pancreatic phospholipase A_2 by interaction with phospholipid vesicles. Aarsman et al. [14] showed that the hydrolysis of *Escherichia coli* membrane phospholipids by pancreatic PLA2 was inhibited by lipocortin from human monocytes in a substrate dependent manner. In both cases inhibition was completely overcome at higher substrate concentrations. Lipocortin also inhibited partially purified preparations of two intracellular phospholipases A_2 isolated from rat liver mitochondria and rat platelets. However, again the inhibition decreased with increasing substrate concentrations [14,15]. From these results it was concluded that lipocortin interacts with the substrate instead of the enzyme.

Sedimentation equilibrium analysis of mixtures of five lipocortin-related phospholipase A_2 inhibitors from human placenta and pancreatic phospholipase A_2 indicated weak association between enzyme and inhibitor ($K_d \geq 3 \times 10^{-5}$ M), insufficient to account for the observed inhibition of enzymatic activity [16]. According to these authors, their results exclude all mechanisms for inhibition in vitro involving complex formation between enzyme and inhibitor.

Recently, experiments with synthetic oligopeptides corresponding to a region of high amino acid similarity between uteroglobin, a steroid induced rabbit secretory protein, and lipocortin I, showed that these peptides have potent phospholipase A_2 inhibitory activity in vitro and striking anti-inflammatory effects in vivo [17]. Almost complete inhibition in vitro is reached with stoichiometrical amounts of peptide to PLA2. The authors suggest that the inhibitory effect of the peptide is exerted through an interaction with the enzyme rather than with the substrate.

2. MATERIALS AND METHODS

N^{α}-*t*-Boc protected amino acids were purchased from Du Pont (USA), trifluoroacetic acid from Pierce (USA), *N,N*-dicyclohexylcarbodiimide and *N,N*-diisopropylethylamine were from Janssen (Belgium) and 1-hydroxybenzotriazole was from Fluka (Switzerland). *N,N*-Dimethylformamide and dichloromethane were from Merck (FRG). Liquid HF was from Matheson (USA), acetonitrile from Baker (The Netherlands). 1-Stearoyl-2-[1'-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (58 mCi mmol^{-1}) was obtained from Amersham (England).

N^{α}-*t*-Boc-leucine resin was prepared by esterification of N^{α}-*t*-Boc-leucine to chloromethylated polystyrene resin 1% cross-linked with divinylbenzene (S-XI, Bio-Rad; 1.34 mM Cl/g) according to [18].

1,2-Dioctanoyl-*sn*-glycero-3-phosphocholine [19] and *rac*-2,3-bis-(hexanoylthio)-propylphosphocholine [20] were synthesized as described. 1,2-Didodecanoyl-*sn*-glycero-3-phosphoglycol was prepared according to De Haas et al. (to be published).

Porcine pancreatic prophospholipase A_2 was purified and converted into phospholipase A_2 (1240 U/mg) as described [21].

2.1. *Synthesis of the peptides His-Asp-Met-Asn-Lys-Val-Leu-Asp-Leu* (PI) *and Met-Gln-Met-Asn-Lys-Val-Leu-Asp-Leu* (PII)

The syntheses of the nonapeptides were performed with a semi-automated peptide synthesizer from Bachem (Switzerland), according to [22]. During the synthesis, the side chains of aspartic acid, lysine and histidine were protected with the O-Bu^t, Cl_2-Z and Tosyl moiety, respectively. After final deprotection of the peptides and cleavage from the resin by liquid HF [22], the peptides were purified by reversed phase HPLC (Pharmacia) using a C18 preparative column (Fluka). The peptides were pure, as judged by analytical reversed phase HPLC (C18 column) and amino acid analysis (LKB, England).

2.2. *Ultraviolet-difference spectroscopy*

The affinity of phospholipase A_2 for the synthetic peptides PI and PII was determined by the procedure described in [23] in a buffer containing 0.1 M Tris-HCl, pH 8, 0.1 M NaCl and 1 mM $CaCl_2$. The enzyme solution was titrated with the peptides until a 25-fold molar excess of the peptides was reached. UV-difference spectra were recorded from 265 to 340 nm.

2.3. *Kinetic measurements*

The egg yolk assay was carried out according to the modified procedure described in [24]. Kinetic measurements using micellar 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine and monomeric *rac*-2,3-bis(hexanoylthio)-propyl-phosphocholine were performed as described [20,25]. The assay of porcine pancreatic phospholipase A_2 on 1-stearoyl-2-[1'-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine was carried out as described [17]. The amount of liberated labelled fatty acid was determined by a modified Dole extraction after 4 min reaction time at 37°C [14]. Before assaying PLA2 activity the enzyme was preincubated with varying amounts of the synthetic peptides PI and PII in a buffer containing 0.1 M Tris-HCl, pH 8, 0.1 M NaCl and 1 mM $CaCl_2$ at 37°C for 10 min as described [17].

2.4. *Monolayer experiments*

The zero-order trough with two compartments and the surface barostat technique used to measure substrate hydrolysis were identical with those described in [26] except that the reaction compartment was 100 ml. The apparatus was from KSV (Finland). Calculation of the velocity of the enzyme reaction was performed as described [27]. The surface pressure (π) was measured by the Wilhelmy plate method.

3. RESULTS AND DISCUSSION

Recently Miele et al. [17] showed that synthetic oligopeptides corresponding to a region of high

Table 1

Amino acid composition of the purified synthetic nonapeptides PI and PII

Amino acid	Peptide I	Peptide II
Asx	3.0 (3)	2.1 (2)
Glx	-	0.9 (1)
Val	1.0 (1)	1.0 (1)
Met	0.9 (1)	2.1 (2)
Leu	2.1 (2)	2.0 (2)
His	0.9 (1)	-
Lys	1.0 (1)	1.0 (1)

The expected amount of each amino acid is shown in parentheses

amino acid sequence similarity between uteroglobin and lipocortin I have potent phospholipase A_2 inhibitory activity in vitro and anti-inflammatory effects in vivo. We synthesized the most potent PLA2 inhibitory peptides PI derived from lipocortin I, residues 246–254 (His-Asp-Met-Asn-Lys-Val-Leu-Asp-Leu) and PII from uteroglobin, residues 39–47 (Met-Gln-Met-Asn-Lys-Val-Leu-Asp-Leu). PI corresponds with peptide P2 [17] and PII corresponds with P3, except that in PII the C-terminal serine is substituted by a leucine, which does not affect the inhibitory potential [17].

The nonapeptides were synthesized by conventional solid phase peptide synthesis methods [28]. Amino acid analysis of the purified peptides gave the desired amino acid compositions (table 1). To determine whether the synthetic peptides PI and PII show inhibition of extracellular pancreatic phospholipase A_2 activity this was measured in five different kinetic assays. In the egg yolk assay PLA2 acts on long chain lecithins present as negatively charged mixed micelles with sodium deoxycholate (DOC). From table 2 it can be seen that in this assay both synthetic peptides, even when present in large excess, do not inhibit phospholipase A_2 activity within the experimental error. In order to establish whether the peptides PI and PII show inhibition of PLA2 activity we also used micelles of 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine as a substrate. Also in this much more sensitive assay both peptides show no inhibitory effect on PLA2 activity (table 2). In the same assay 1-acyl-2-acylamino-*sn*-glycero-3-phosphocholine, a potent transition state analogue, shows considerable inhibition of phospholipase A_2 activity [3].

It is known that pancreatic phospholipase A_2 also possesses enzymatic activity toward substrate present as monomers [20] although considerably lower as compared to micellar substrate. Using 0.5 mM *rac*-2,3-bis(hexanoylthio)-propylphosphocholine (CMC is 1 mM) as monomeric substrate, it can be seen from table 2 that both peptides I and II have no effect on PLA2 activity in this assay,

Table 2

PLA2 inhibitory activity of the synthetic nonapeptides PI and PII in three different kinetic assays

Assay	Peptide	Peptide:PLA2	PLA2 residual activity (%)
Egg yolk[a]	PI	300:1	90
	PII	100:1	95
1,2-Dioctanoyl-*sn*-glycero-3-phosphocholine[b]	PI	450:1	98
	PI	10:1	99
	PII	450:1	98
rac-2,3-Bis(hexanoylthio)-propylphosphocholine[c]	PI	400:1	100
	PI	2000:1	100
	PII	200:1	100
	PII	1000:1	100

[a] The egg yolk assay was performed as described in section 2 at pH 8, 40°C, in the presence of DOC

[b] The 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine assay was performed at 45°C, in 0.01 M Tris-HCl, 0.1 M NaCl, 1 mM $CaCl_2$ (pH 8)

[c] The *rac*-2,3-bis(hexanoylthio)-propylphosphocholine assay was performed at 25°C in 0.1 M Tris-HCl, 0.1 M NaCl and 1 mM $CaCl_2$ (pH 8)

The % residual phospholipase A_2 activity was calculated from the specific activities of PLA2 measured under the same conditions in the presence and absence of the peptides

which indicates that the active site of the enzyme is not involved in any inhibition event.

To answer the important question if the lipocortin derived peptides exert their action on PLA2 only when the substrate contains an arachidonic acid on the *sn*-2 position, the peptides PI and PII were tested in 50 nM concentrations in the in vitro assay under the assay conditions in [17].

As can be seen from table 3 phospholipase A_2 present in an almost one-to-one ratio of enzyme (36 nM) to peptide (50 nM) releases similar amounts of [1'-^{14}C]arachidonic acid as compared to PLA2 without preincubation with the peptide. Also an almost 30000-fold excess of peptide (1 mM) present during preincubation with the enzyme, does not result in a detectable inhibition of PLA2.

Finally, monolayer experiments were performed to check if a large excess of peptide is able to prevent penetration into and hydrolysis of the negatively charged substrate 1,2-didodecanoyl-*sn*-glycero-3-phosphoglycol. Before injection of PLA2 in the reaction compartment, 0.5 mg (4 μM) peptide was injected, which did not result in an increase of the surface pressure. At a surface pressure of 30 dynes/cm, the hydrolytic activity of PLA2 (0.28 nM) in the absence of peptide was 13.0 mm/min per μg protein injected. In the presence of the synthetic peptides PI and PII, PLA2 reached, in a similar experiment, a velocity of 13.3 mm/min per μg protein. These results show that there is no significant inhibitory influence of the peptides PI and PII on the hydrolytic and penetrating properties of phospholipase A_2.

In addition direct binding studies using UV-difference spectroscopy were performed to detect possible interactions between porcine pancreatic PLA2 and each of these peptides. It is well known that upon binding of monomeric substrate analogues to PLA2 difference spectra are generated originating from Tyr^{69} perturbation close to the active site [29]. Similarly interaction of micellar substrate analogues with PLA2 results in difference spectra typical for Tyr^{69} and Trp^{3} perturbation, residues known to be part of the lipid binding domain (LBD) [29]. Titration of either peptide PI or PII to PLA2 up to a 25-fold molar excess does not give rise to any difference spectrum, strongly suggesting that the peptides have no affinity for PLA2 (not shown). However, an interaction of the peptides with non-aromatic residues, e.g. by charge interaction cannot be excluded.

Table 3

PLA2 inhibitory activity of the synthetic nonapeptides PI and PII on 1-stearoyl-2-[1'-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (PC)

Assay conditions[a]	Percentage radioactivity liberated by PLA2
10 μM PC, no PLA2	0.2
10 μM PC, 36 nM PLA2	24
10 μM PC, 36 nM PLA2, 50 nM PI	32
10 μM PC, 36 nM PLA2, 1 mM PI	33
10 μM PC, 36 nM PLA2, 50 nM PII	24
10 μM PC, 36 nM PLA2, 1 mM PII	23
10 μM PC, 1.4 μM PLA2	70

[a] The assay was performed in a final volume of 50 μl in the buffer described in [17], containing 1 mM DOC. The DOC was not present during the preincubation of the enzyme with the peptides

It is obvious from these results that the peptides PI and PII do not inhibit phospholipase A_2 activity in any in vitro assay after preincubation of the enzyme with the peptides. This strongly supports the results recently reported by Ahn et al. [16], in which the affinity between enzyme and lipocortin was measured. The reported K_d value excludes all mechanisms for inhibition in vitro involving complex formation between enzyme and inhibitor [16]. Therefore it is unlikely that the peptides PI and PII derived from lipocortin I are inhibitors, acting by binding to the enzyme, as suggested by Miele et al. [17]. The results also do not support the model which proposes the binding of the inhibitor to the substrate instead of to the enzyme [12–15], as the mechanism of lipocortin action. Even when only a 1.4-fold molar excess of peptide is present over the substrate in the *rac*-2,3-bis(haxanoylthio)-propylphosphocholine assay, no decrease in PLA2 activity was observed (table 2). This finding confirms the data obtained with monolayer experiments: both peptides do not interact with phospholipids in such a way that phospholipase A_2 activity is inhibited.

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