

# Acylchain Specificity and Kinetic Properties of Phospholipase A<sub>1</sub> and A<sub>2</sub> of Bone Marrow-Derived Macrophages

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Phospholipase A<sub>1</sub>/A<sub>2</sub>, Macrophages, Arachidonic Acid, Acylchain Specificity

The fatty acyl specificity of phospholipase A<sub>1</sub> and A<sub>2</sub> in homogenates of mouse bone marrow-derived macrophages was determined using phosphatidylcholine and phosphatidylethanolamine of different acylchain composition. Phosphatidylcholine with arachidonoyl at position 2 was cleaved preferentially by an alkaline phospholipase A<sub>2</sub> (pH-optimum 9.0) leading to selective liberation of arachidonic acid. In contrast, phosphatidylcholines with oleoyl or linoleoyl at position 2 were degraded mainly by an acid phospholipase A<sub>1</sub> (pH-optimum 4–5) resulting in a conservation of these fatty acids esterified in lysophosphatides. Substrate kinetics of the alkaline phospholipase A<sub>2</sub> revealed a 30 fold higher affinity ( $K_m = 3.8 \times 10^{-7}$  M) for 1-acyl-2-arachidonoyl-glycerophosphocholine compared to 1-acyl-2-oleoyl-glycerophosphocholine. The kinetic data were not influenced by endogenous lipids indicating that exogenous substrates do not equilibrate with cellular lipids.

These results are suitable to explain a selective liberation of arachidonic acid from a mixture of phospholipids.

## Introduction

Macrophages exhibit high activities of phospholipases and are besides many other cells potent producers of arachidonic acid and its metabolites. It has been postulated that the hydrolytic liberation\* of arachidonic acid from phospholipids is the limiting step for its further metabolism [1, 2] but it is still a matter of controversy whether cellular phospholipase A exhibits specificity for distinct fatty acids. For example, in cases where liberation of arachidonic acid is assumed to be caused by phospholipase A<sub>2</sub> cleaving PC one must postulate an enzyme with distinct specificity for 1-acyl-2-arachidonoyl-glycerophosphocholine. Alternative mechanisms which may also result in a preferential accumulation and release\* have been discussed previously [3] and are

based on an inhibition of a specific lysophosphatide acyltransferase which catalyzes the reacylation of lysophosphatides. Evaluation of specificities for distinct acyl moieties requires the use of defined molecular species. These are now available as specifically labeled synthetic compounds which have been used here in a comparative study. However, even under defined assay conditions the determination of enzyme activities is questionable because non-purified enzymes (homogenates) contain cellular lipids. Even if the exact composition of cellular lipids is known this does not necessarily facilitate the analysis because it is not guaranteed that the added substrate equilibrates with the endogenous lipid.

The analysis of the positional specificity is further complicated by interfering enzymes in particular such as lysophospholipases. The uncertainties in the enzyme determination are of particular importance for questions related to the control of arachidonic acid metabolism.

Evidence for preferential accumulation of arachidonic acid is mainly based on experiments with intact cells ([4–6], for review see [7]).

There are only a few reports which provide evidence for acyl chain specificity of phospholipase A and most of these are based on experiments with platelets [8, 9]. In macrophages arachidonic acid-liberating phospholipase A<sub>2</sub> activities of different pH-optima were detected but no comparative data were reported with respect to acyl chain specificities [10].

\* We use the definitions of the terms “liberation” and “release” of arachidonic acid according to the proposal of Irvine [7]. Liberation means hydrolytic cleavage of fatty acids from its esterified form and “release” is used for its extrusion into the extracellular space.

**Abbreviations:** GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

**Enzymes:** Lysophospholipase or lysolecithin acylhydrolase (EC 3.1.1.5); phospholipase A<sub>1</sub> or phosphatide-1-acylhydrolase (EC 3.1.1.32); phospholipase A<sub>2</sub> or phosphatide-2-acylhydrolase (EC 3.1.1.4).

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We have attempted to address the problems discussed above by comparing phospholipase A<sub>1</sub> and A<sub>2</sub> activities using substrates of different acyl chain composition. Enzyme kinetics and the determination of affinities was carried out using both cell-homogenate preparations containing lipid and homogenates with almost no endogenous lipid. Possible interference by lysophospholipases was excluded by using assays which allowed their selective inhibition.

## Materials and Methods

### *Bone marrow cultures*

Six to eight week-old C57BL/6 female mice were obtained from our own breeding colony. Cells were flushed from the femora and cultivated in the high glucose formulation of Dulbecco's modified Eagle's medium (Gibco, Karlsruhe, FRG) supplemented with 10% fetal calf serum, 5% horse serum, 1 mM sodium pyruvate (Gibco, Karlsruhe, FRG),  $5 \times 10^{-5}$  M  $\beta$ -mercaptoethanol (Roth, Karlsruhe, FRG), 40 U/ml penicillin and 2  $\mu$ g/ml amphotericin B (Sigma, München, FRG). The culture medium contained 30% L-cell conditioned medium and resulted in a pure macrophage population [11].

$3 \times 10^7$  bone marrow cells ( $6.5 \times 10^4$  cells/ml) were cultured in hydrophobic teflon film bags (Heraeus, Hanau, FRG) at 37 °C and a humidified atmosphere containing 10% CO<sub>2</sub> [12, 13]. The teflon bags were prepared by using a sealer (polystar 401 M-RPA, Rische und Herfurth, Hamburg, FRG) and were sterilized with ethylenoxide.

After 10 days of culture the cells were harvested from the culture bags by gently shaking the culture containers at room temperature for 5 min. Then the cells were collected by centrifugation at  $300 \times g_{av}$  at 10 °C for 10 min. Cell viability always exceeded 90% as checked by trypan blue exclusion. Cell homogenates were prepared in 250 mM sucrose/10 mM HEPES (pH 7.4) by freezing and thawing in liquid nitrogen followed by passing the suspension through a No. 18 needle (ID 0.45 mm).

### *Preparation of L-cell conditioned medium*

L-cells (strain L 929 S) were maintained in culture in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in Nunc 75-cm<sup>2</sup> tissue culture flasks (Nunc, Wiesbaden, FRG). Conditioned medium was prepared in hydrophilic teflon film bags

by seeding  $1 \times 10^5$  cells/ml into Dulbecco's modified Eagle's medium containing 10% fetal calf serum. After one week of incubation, at 37 °C under a humidified atmosphere containing 10% CO<sub>2</sub>, the culture supernatant was harvested. The conditioned medium was centrifuged at  $200 \times g_{av}$  for 15 min at 4 °C, sterilized by filtration and kept at -20 °C until use.

### *Determination of phospholipase A<sub>1</sub> and A<sub>2</sub> activity*

Enzyme activities were determined using phosphatidylcholines or phosphatidylethanolamines specifically labeled in position 2 with [1-<sup>14</sup>C]arachidonic, or [1-<sup>14</sup>C]linoleic, or [1-<sup>14</sup>C]oleic acid (New England Nuclear). Standard assay mixtures (500  $\mu$ l) contained 0.1 M acetate-buffer (pH 3–6) or 0.1 M Tris-buffer (pH 7–9), 10–50  $\mu$ g of homogenate protein and 0.02  $\mu$ Ci (0.38 nmol) phosphatidylcholine (ethanolamine) as substrate. The assays were carried out in the presence and absence of 4 mM CaCl<sub>2</sub>. Substrate mixtures were prepared without detergent by brief sonication under nitrogen (Branson sonifier, setting 1 for 1 min). After an incubation time of 15 to 60 min at 37 °C the reaction was terminated by the addition of 5.0 ml of methanol. The lipids were extracted and separated by thin-layer chromatography as described below. The radioactivity of generated lysophospholipid was used for the calculation of phospholipase A<sub>1</sub> activity and the radioactivity of free fatty acids for phospholipase A<sub>2</sub> activity. Alternatively, phospholipase A<sub>2</sub> was determined using selective extraction of liberated free fatty acids by the "heptane-silica"-method (see below).

### *Extraction, chromatography and quantitation of lipids*

Lipid extraction of total lipids, chromatography and measurement of labeled lipids was carried out as described previously [14]. In addition, liberated fatty acids also were selectively extracted by the "heptane-silica"-method with Dole's reagent [15] followed by adsorption of phospholipids on silica in a modified procedure according to Sundaram *et al.* [16]. Control experiments showed a recovery of fatty acids exceeding 96% and a contamination with lipid substrates being less than 0.1%.

The total phospholipid content was measured by phosphorus determination in purified extracts according to the method of Lowry *et al.* [17].

## Results

### *Assay conditions for the determination of phospholipase A<sub>1</sub> and A<sub>2</sub> activity*

The dependence of the reaction velocity on protein concentration and incubation time was studied using the standard assay. Fig. 1 and 2 show that linearity can be achieved in a limited range up to 60 min incubation time and 30  $\mu$ g protein of macrophage homogenates. At the endpoints of the linear range about 15% of the substrate is hydrolyzed.

### *pH-optima and acyl chain specificity*

The pH-optima of phospholipase A<sub>2</sub> of bone marrow-derived macrophages (Fig. 3a, b) are similar to

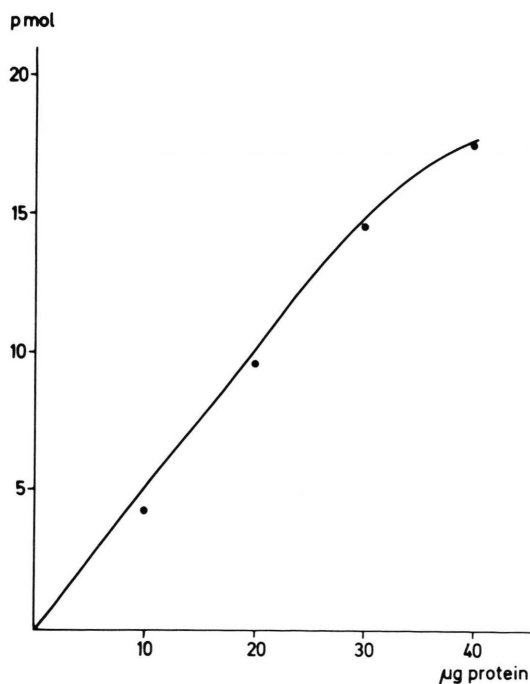


Fig. 1. Phospholipase A<sub>2</sub> activity with increasing concentrations of macrophage homogenate. Assays were performed in 500  $\mu$ l 0.1 M Tris buffer pH 9.0 containing as substrate 380 pmol (0.02  $\mu$ Ci) 1-palmitoyl-2-[<sup>14</sup>C]arachidonoylglycerophosphocholine, 2  $\mu$ mol CaCl<sub>2</sub> and 10 to 40  $\mu$ g of macrophage homogenate protein. The reaction mixtures were incubated for 15 min at 37 °C. Values are expressed as pmol liberated arachidonic acid per assay and were calculated from the radioactivity of free arachidonic acid after separation by thin-layer chromatography or by the heptan-silica method. For details see Materials and Methods.

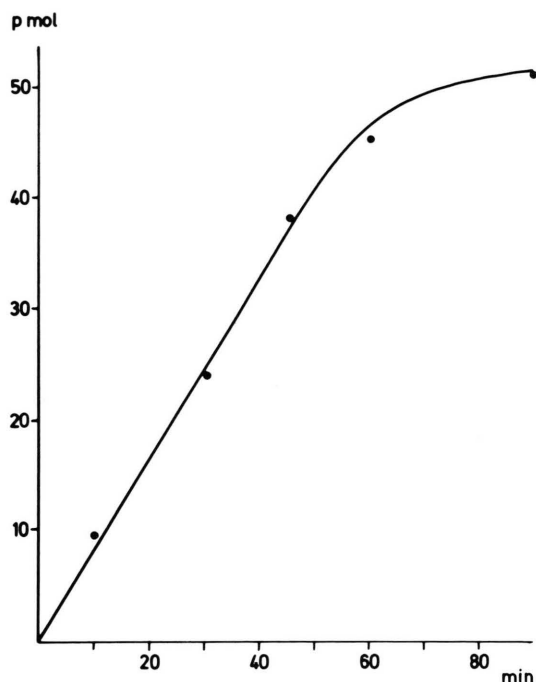
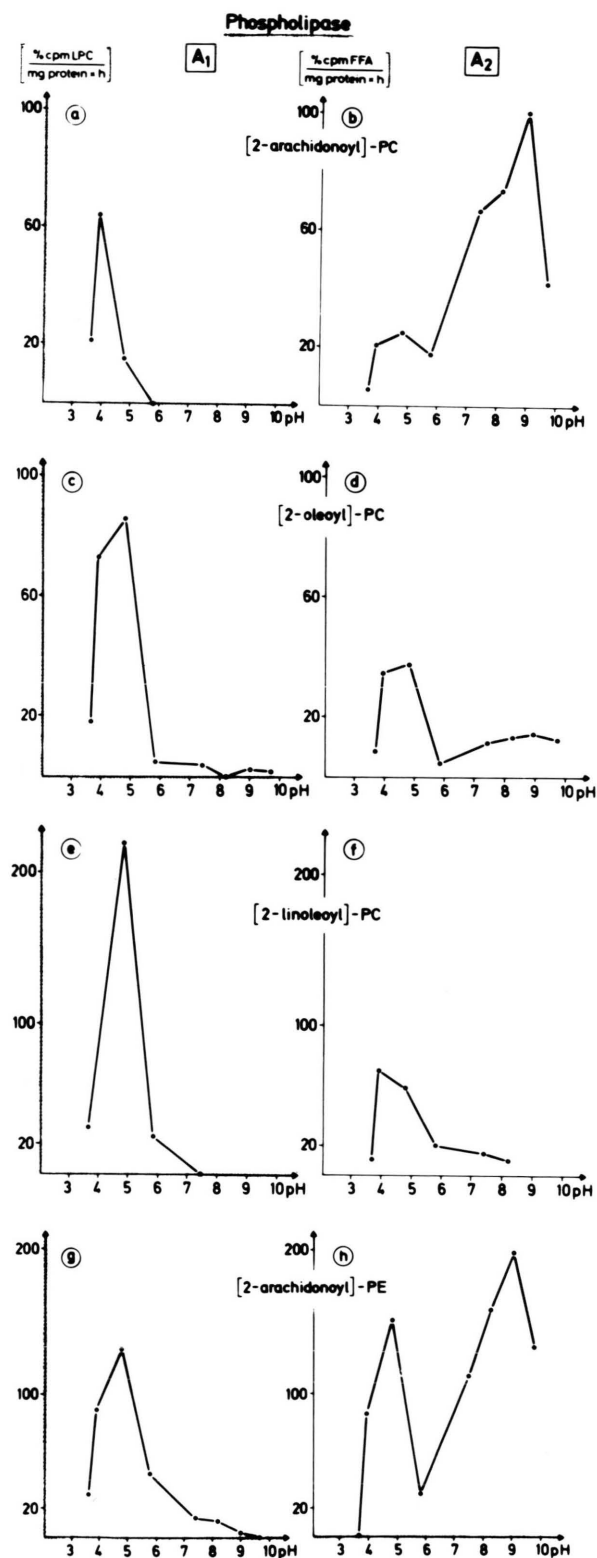


Fig. 2. Time dependence of phospholipase A<sub>2</sub> reaction. Assays were performed in 500  $\mu$ l 0.1 M Tris buffer pH 9.0 containing 380 pmol (0.02  $\mu$ Ci) 1-palmitoyl-2-[<sup>14</sup>C]arachidonoylglycerophosphocholine, 2  $\mu$ mol CaCl<sub>2</sub> and 20  $\mu$ g of macrophage homogenate protein. For further details see Fig. 1 and Materials and Methods.

those reported for resident peritoneal macrophages [10]. Using 1-acyl-2-arachidonoyl-GPC as substrate, a smaller peak was detected at pH 4–5 and a more prominent one at pH 8–9 (Fig. 3b). In addition we found high activities of phospholipase A<sub>1</sub> with an pH-optimum at pH 4–5. The most striking feature concerns the acyl chain specificity, in particular of the alkaline phospholipase A<sub>2</sub>. This enzyme preferentially cleaves 1-acyl-2-arachidonoyl-GPC whereas PC's containing oleoyl or linoleoyl moieties in position 2 are predominantly degraded by acid phospholipases, mainly acid phospholipase A<sub>1</sub>. Interestingly this acyl chain specificity is not restricted to PC but is also revealed if PE is used as substrate. Fig. 3h shows that the relative activities and pH-optima using 1-acyl-2-arachidonoyl-GPE as substrate are similar to those obtained with 1-acyl-2-arachidonoyl-GPC (Fig. 3b).

The Ca<sup>++</sup> dependence was found to be similar as reported for resident peritoneal macrophages [10] *i.e.* the acid activities (A<sub>1</sub> and A<sub>2</sub>) being inhibited and the alkaline activity being activated by Ca<sup>2+</sup>.



### Substrate kinetics

Using 3 PC's differing in the acyl moieties in position 2, substrate kinetics of the alkaline phospholipase  $A_2$  were carried out. Moreover a comparison was made between 2 different enzyme preparations; a total homogenate of macrophages and a supernatant at ( $177,000 \times g$  for 60 min) which was almost free of phospholipids. As depicted in Fig. 4 and Table 1 great differences were obtained which resulted in a 30-fold higher affinity for 1-acyl-2-arachidonoyl-GPC compared to 1-acyl-2-oleoyl-GPC. Also the absolute  $K_m$ -value for 1-acyl-2-arachidonoyl-GPC with  $K_m = 3.8 \times 10^{-7} M$  reveals an unexpected high affinity-enzymes.

Interestingly both enzyme preparations showed very similar kinetics and resulted in similar affinities and maximal velocities (Table 1). For comparison specific activities, as measured under standard assay conditions (0.38 nmol substrate/assay), are included in Table 1.

### Effects of deoxycholate on the activities of lysophospholipase and phospholipase $A_2$

As shown for many other cell types deoxycholate effectively inhibits the lysophospholipase [18]. Therefore addition of this detergent is an important method for excluding the possibility that cleavage at position 2 is due to the combined action of phospholipase  $A_1$  and lysophospholipase. Fig. 5 shows that macrophage homogenates exhibit high activities of lysophospholipase with an pH-optimum at pH 8.3. This enzyme is almost completely inhibited by the addition of deoxycholate (0.05 mg/ml) in the presence of albumin (4 mg/ml). These conditions which are similar to those reported by Hsueh *et al.* [19] were chosen to measure phospholipase  $A_2$  activity.

Fig. 3. pH dependence of phospholipase  $A_1$  and  $A_2$ . Four different substrates were used; phosphatidylcholine with 3 different fatty acyl moieties in position 2: [ $^{14}C$ ]arachidonoyl, [ $^{14}C$ ]oleoyl, and [ $^{14}C$ ]linoleoyl and phosphatidylethanolamine with [ $^{14}C$ ]arachidonoyl. Reaction mixtures (500  $\mu$ l) contained either 0.1 M acetate buffer (pH 3–6) without  $Ca^{2+}$  or 0.1 M Tris buffer (pH 7–9) with the addition of 2  $\mu$ mol  $CaCl_2$ . 20  $\mu$ g of macrophage homogenate protein were used per assay. Reaction mixtures were incubated for 60 min at 37  $^{\circ}C$ . For further conditions see Fig. 1 and Materials and Methods.

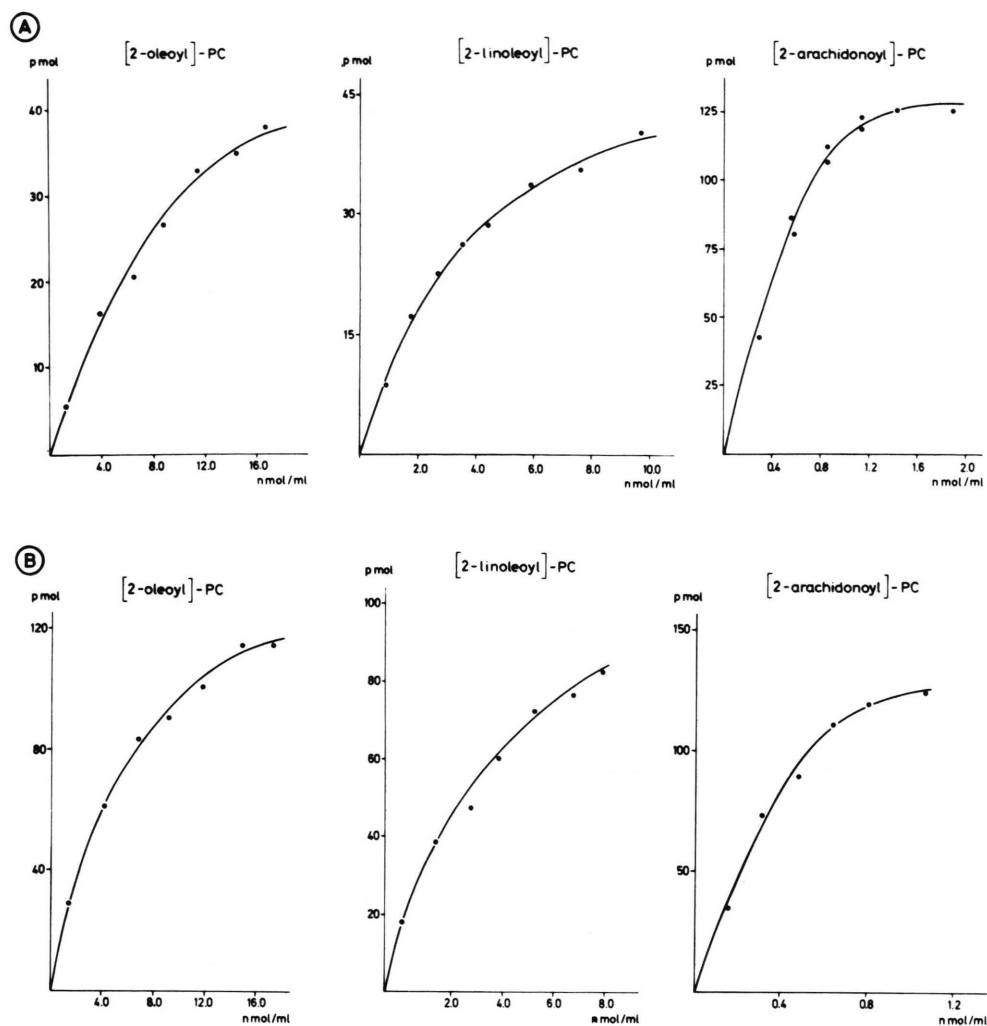


Fig. 4. Substrate kinetics of phospholipase  $A_2$ . Three phosphatidylcholines differing in the fatty acyl moieties at position 2 ( $[^{14}C]$ oleoyl,  $[^{14}C]$ linoleoyl or  $[^{14}C]$ arachidonoyl) were used as substrates.

Enzyme source: A: Macrophage homogenate (10–30  $\mu$ g protein per assay) containing 200 nmol endogenous phospholipid/mg protein.

B: Membrane-free supernatant ( $177,000 \times g_{av}$ ) from macrophage homogenate (10–30  $\mu$ g per assay) containing 6 nmol endogenous phospholipid/mg protein. Assays were performed in 500  $\mu$ l 0.1 M Tris buffer pH 9.0 containing 2  $\mu$ mol  $CaCl_2$ . Reaction mixtures were incubated for 60 min at 37  $^{\circ}C$ .

Table I. Michaelis constants and maximal velocities of phospholipase  $A_2$ . Kinetic parameters were obtained from experiments given in Fig. 4. Specific activities refer to experiments under standard assay conditions.

Substrate		$K_m$ [ $\mu M$ ]	$V_{max}$ [ $pmol \times mg^{-1} \times min^{-1}$ ]	Specific activity [ $pmol \times mg^{-1} \times min^{-1}$ ]
1-palmitoyl-2-oleoyl-GPC	homogenate	13.0	15	0.9
	supernatant	9.0	58	9.0
1-palmitoyl-2-linoleoyl-GPC	homogenate	5.3	22	1.6
	supernatant	5.1	29	4.0
1-palmitoyl-2-arachidonoyl-GPC	homogenate	0.38	110	65.0
	supernatant	0.70	167	84.0



Fig. 6 shows that under these limited conditions the cleavage of arachidonoyl moieties was not reduced. Moreover, separation of products by thin-layer chromatography revealed that no labeled lysophosphatide was generated (data not shown). These results indicate that the cleavage of arachidonoyl moieties at pH 9 is exclusively catalyzed by phospholipase A<sub>2</sub>. With the exception of experiments confirming the involvement of phospholipase A<sub>2</sub>, detergents were not used in phospholipase A assays. In the presence of deoxycholate, cholate or Triton X-100, fatty acid specificities of the phospholipases are altered and the enzyme activity is impaired (data not shown).

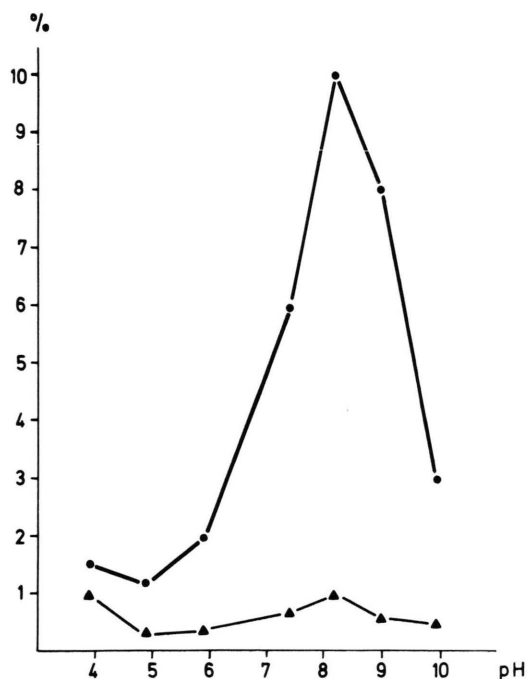


Fig. 5. pH dependence of lysophospholipase and inhibition by deoxycholate (DOC). The assay conditions correspond to those used for phospholipase A<sub>2</sub> determination (standard assay). Assays were performed in 500  $\mu$ l 0.1 M acetate buffer (pH 4–6 without Ca<sup>2+</sup>) or 0.1 M Tris buffer (pH 7–10) with the addition of 2  $\mu$ mol CaCl<sub>2</sub>. [1-<sup>14</sup>C]palmitoyl-glycerophosphocholine (0.1  $\mu$ Ci, 1.8 nmol) was used as substrate and assays which contained 20  $\mu$ g of macrophage homogenate protein were performed with and without the addition of deoxycholate (0.025 mg/assay) and albumin (2 mg/assay). Reaction mixtures were incubated for 60 min at 37 °C. Enzyme activities are expressed as percentage liberation of [<sup>14</sup>C]palmitic acid from the substrate.

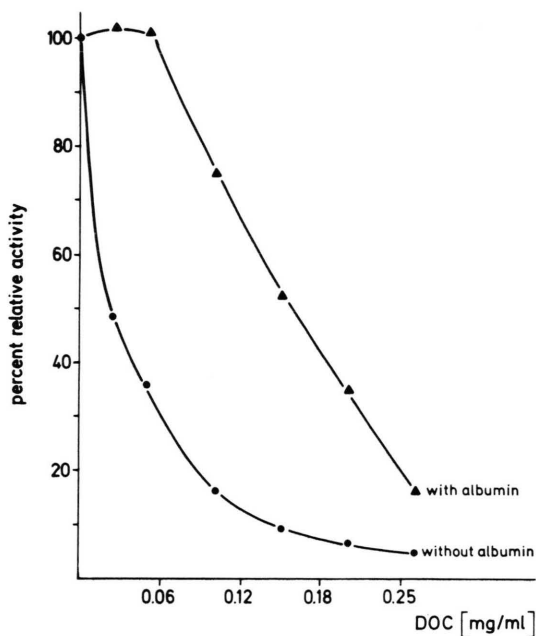


Fig. 6. Inhibition of phospholipase A<sub>2</sub> by deoxycholate (DOC). Assays were performed in 500  $\mu$ l 0.1 M Tris buffer pH 9.0 containing as substrate 380 pmol (0.02  $\mu$ Ci) 1-palmitoyl-2-[<sup>14</sup>C]arachidonoylglycerophosphocholine, 2  $\mu$ mol CaCl<sub>2</sub>, 25  $\mu$ g of macrophage homogenate protein and with or without deoxycholate (0.012–0.125 mg/assay) and albumin (2 mg/assay). The reaction mixtures were incubated for 60 min at 37 °C.

## Discussion

Macrophages are potent producers of arachidonic acid metabolites [4, 20]. The regulation and control of the obligatory step of this pathway, *i.e.* the liberation of arachidonic acid, is still a matter of discussion [3, 7]. Macrophages of different origin exhibit relatively high activities of phospholipase A<sub>2</sub>. Since alternative pathways such as the degradation of phosphatidylinositol by phospholipase C, which operate in platelets [21, 22] do not lead to liberation of fatty acids in macrophages [3, 23], phospholipase A<sub>2</sub> has to be considered as the reaction responsible for the liberation of acyl moieties.

However, a major problem concerns the specificity of phospholipase A for distinct fatty acids. Preferential release of arachidonic acid from cells has been detected in platelets [5]. Also in these cells, the direct determination of arachidonic acid liberation from phospholipids revealed evidence for acyl-specific phospholipases [8, 9]. Macrophages of different origin degrade arachidonic acid-containing

phospholipid [3, 10, 19, 23], however, no comparisons have previously been carried out using substrates with different fatty acids. With respect to the degradation of 1-acyl-2-arachidonoyl-GPC, our results are generally consistent with those obtained with resident peritoneal macrophages by Wightman *et al.* [10]. Two phospholipase A<sub>2</sub> activities were detected with pH-optima at 4–5 and 9. In contrast to Wightman *et al.* [10] we detected in bone marrow-derived macrophages, high activities of phospholipase A<sub>1</sub> at pH 4–5 which for all substrates were higher than the corresponding phospholipase A<sub>2</sub> activity.

Most interesting is the fact that the alkaline phospholipase A<sub>2</sub> preferentially degraded arachidonoyl phospholipids (PC and PE). In contrast substrates containing oleic or linoleic acid in position 2 were preferentially hydrolyzed by acid phospholipase A<sub>1</sub>.

Thus mainly arachidonic acid is liberated from a phospholipid mixture and degradation of other lipids containing different fatty acids by phospholipase A<sub>1</sub> does not lead to their liberation but to their conservation esterified in phospholipids. The low ability of the alkaline phospholipase A<sub>2</sub> to degrade linoleic acid containing phospholipids also was observed by Derksen and Cohen [9] in platelets and thus seems to be a general feature of this enzyme. A second problem concerns the interference of enzymes metabolizing products of the phospholipase A reaction in particular those metabolizing lysophosphatides. In intact cells one major route is the reacylation of lysophosphatides. In a previous paper [24] we showed that in homogenates of macrophages no reacylation takes place unless ATP (and CoA) is added. Therefore this reaction can be excluded to be active in our assay systems. Degradation of generated lysophosphatides by lysophospholipases, however, is active and may lead to severe problems concerning the classification of phospholipase A<sub>1</sub> or A<sub>2</sub> activity. Since these difficulties can not be completely overcome by double labeled substrates, the effective inhibition of lysophospholipases by deoxycholate offered a method to differentiate between combined reactions and those solely due to phospholipase A<sub>2</sub>. We provide evidence that the alkaline (pH 9) activity is exclusively caused by phospholipase A<sub>2</sub> since also under conditions where the lysophospholipase was inhibited no accumulation of labeled lysophosphatide was observed. The combined addition of deoxycholate and albumin as used by Hsueh *et al.*

[19] offers advantages because, within a limited concentration range, deoxycholate can be used without inhibition of the phospholipase A<sub>2</sub>. However, we found that a general use of this system is questionable as specificities are altered. Therefore we avoided addition of detergents in standard assays and also in kinetic studies.

A third problem concerns the fact that the enzyme source contains endogenous lipid and its mode of equilibrium with exogenous substrate is unknown. We have attempted to address this problem by using two different enzyme preparations *i.e.* total homogenate and a soluble supernatant fraction containing almost no lipid. The kinetic assays using increasing concentrations of substrates interestingly resulted in very similar  $K_m$  values and also the shape of the curves was almost identical. This indicates that the added substrate does not equilibrate with endogenous lipids and therefore  $V_{max}$  and  $K_m$  values seem to be reliable. On the other hand we found that the phospholipase A<sub>2</sub> specific for 1-acyl-2-arachidonoyl-GPC was not only inhibited by ionic detergents (deoxycholate) but also by nonionic detergents (Triton X-100) as also reported by Wightman *et al.* [10]. According to the criteria of Dawson [25], this indicates that the specificity of the enzyme is not exclusively due to properties of the enzyme and therefore the kinetic parameters should be classified as “apparent” values. Besides these limitations the properties of the alkaline phospholipase A<sub>2</sub>, with respect to its activity and specificity, are suitable to explain a selective liberation of arachidonic acid from PC (and PE) in intact macrophages.

Moreover, as shown previously [26], liver macrophages exhibit an alkaline phospholipase A<sub>2</sub> of similar specificity which is activated in cells stimulated with lipopolysaccharide.

The data presented do not rule out the possibility that under physiological conditions the liberation of arachidonic acid is regulated by additional mechanisms such as controlled reacylation [3] and reversed transfer of acyl moieties [24].

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