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# Calcium-independent phospholipase $A_2$ in rat tissue cytosols

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**Cytosols (105000 × *g* supernatant) from seven rat tissues were assayed for $Ca^{2+}$-independent phospholipase $A_2$ activity with either 1-acyl-2-[1-$^{14}$C]linoleoyl-*sn*-glycero-3-phosphocholine, 1-acyl-2-[1-$^{14}$C]linoleoyl-*sn*-glycero-3-phosphoethanolamine or 1-*O*-hexadecyl-2-[9,10-$^{3}H_2$]oleoyl-*sn*-glycero-3-phosphocholine as substrate. Low but consistent activities ranging from 10–120 pmol/min per mg protein were found in all tissues. The highest activities were present in liver, lung and brain. Total activities in mU/g wet weight were rather constant, ranging from 0.43 (heart) to 1.36 (liver). The soluble enzyme from rat lung cytosol was further investigated and was found to be capable of hydrolyzing microsomal membrane-associated substrates without exhibiting much selectivity for phosphatidylcholine species. Comparative gel filtration experiments of cytosol prepared from non-perfused and perfused lungs indicated that part of the $Ca^{2+}$-independent phospholipase $A_2$ originated from blood cells, but most of it was derived from lung cells. Lung cytosol also contained $Ca^{2+}$-dependent phospholipase $A_2$ activity, a small part of which originated from blood cells, presumably platelets. The major amount of $Ca^{2+}$-dependent phospholipase $A_2$ activity, however, came from lung cells. Neither this enzyme nor the $Ca^{2+}$-independent phospholipase $A_2$ from lung tissue showed immunological cross-reactivity with monoclonal antibodies against $Ca^{2+}$-dependent phospholipase $A_2$ isolated from rat liver mitochondria.**

## Introduction

Phospholipase $A_2$ hydrolyze the acyl ester bond at the *sn*-2-position of glycerophospholipids. Apart from their occurrence in high amounts in venoms and pancreatic secretions [1], these enzymes have been detected in numerous eukaryotic cells and derived subcellular fractions [2], thus emphasizing their ubiquity and importance. Intracellular phospholipases $A_2$ are believed to play an important role in arachidonate release for prostanoid and leukotriene formation [2–6] and in 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine production for biosynthesis of platelet-activating factor [7,8]. In addition, these enzymes have been implicated in cellular phospholipid turnover and deacylation-reacylation remodeling cycles to produce certain phospholipid species [2,9,10] in receptor-mediated transmembrane signalling [11–13] and in cytotoxicity and tissue injury [14]. Consequently, phospholipase $A_2$ activation has been postulated to contribute to the pathophysiology of inflammation and tissue injury in various disease states, such as rheumatoid arthritis, psoriasis, pancreatitis, gastrointestinal disorders, septic shock,

Abbreviation: ELISA, enzyme-linked immunosorbent assay.

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myocardial ischemia and airway hyperreactivity [15].

The extracellular phospholipases $A_2$ from venoms and pancreas are low-molecular-weight proteins of about 15000 Da that require $Ca^{2+}$ for activity [1]. Most of the intracellular phospholipases $A_2$ that have been purified until now share these properties [2,10]. However, an increasing number of $Ca^{2+}$-independent phospholipase $A_2$ activities has been reported. This enzymatic activity was first recognized in rat liver lysosomes [16], where it exhibited and acidic pH optimum, characteristic of lysosomal enzymes. Neutral or alkaline active forms of $Ca^{2+}$-independent phospholipase $A_2$ have since been described in soluble fractions isolated from rat lung [17], chicken erythrocytes [18], canine myocardium [19] and in human amniotic fluid [20]. The latter two enzymes were claimed to show some selectivity for the release of fatty acids from ether glycerophospholipids. A $Ca^{2+}$-independent phospholipase $A_2$ with selectivity for phosphatidylethanolamine was recently described in human platelets and could be separated from a $Ca^{2+}$-dependent enzyme acting on phosphatidylcholine [21]. A phospholipase $A_2$-type of enzymatic activity which specifically hydrolyzes the acetyl ester bond in 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet-activating factor), denoted platelet-activating factor : acetylhydrolase, was detected in plasma and in the cytosolic fraction of a variety of rat tissues and also appeared to be $Ca^{2+}$-independent [22,23]. Highest activities were reported for kidney and lung cytosolic fractions. Platelet-activating factor : acetylhydrolase in rat lung cytosol could be distinguished from the $Ca^{2+}$-independent phospholipase $A_2$ acting on long-chain glycerophospholipids by separation on DEAE-cellulose and differential effects of *p*-bromophenacylbromide and diisopropylfluorophosphate [24]. Membrane-associated forms of $Ca^{2+}$-independent phospholipase $A_2$ activity towards long-chain phospholipids have recently been reported for rabbit neutrophil plasma membranes [25], bovine endothelial membranes [26] and both guinea pig [27] and rat [28] brush-border membranes.

In this paper, we have investigated the presence of $Ca^{2+}$-independent phospholipase $A_2$ activity towards diacylphospholipids in a variety of rat tissue cytosols. To avoid interference in the measured release of *sn*-2-fatty acids by consecutive action of phospholipases $A_1$ and lysophospholipases, the assays were also performed with 1-alkyl-2-[9,10-$^3H_2$]oleoyl-*sn*-glycero-3-phosphocholine. The enzyme from rat lung cytosol was examined for its immunochemical relationship with $Ca^{2+}$-requiring intracellular phospholipases $A_2$ using monoclonal antibodies against rat liver mitochondrial phospholipase $A_2$ [29]. In view of the potential importance of $Ca^{2+}$-independent phospholipases $A_2$ in phospholipid metabolism and arachidonate release under the intracellular conditions of sub-micromolar concentrations of free $Ca^{2+}$, the question of whether such cytosolic proteins can act at all on membrane-embedded substrate was addressed.

## Materials and Methods

*Chemicals.* [9,10-$^3H_2$]Oleic acid was obtained from New England Nuclear, 's-Hertogenbosch, The Netherlands. [1-$^{14}$C]Linoleic acid, 1-acyl-2-[9,10-$^3H_2$]oleoyl-*sn*-glycero-3-phosphocholine, 1-acyl-2-[1-$^{14}$C]palmitoyl-*sn*-glycero-3-phosphocholine and 1-acyl-2-[1-$^{14}$C]arachidonoyl-*sn*-glycero-3-phosphocholine were from Amersham International, Buckinghamshire, U.K. 1-*O*-Hexadecyl-*sn*-glycero-3-phosphocholine was a product of Bachem, Bubendorf, Switzerland. Oleic acid was obtained from Merck, F.R.G. and silica gel Silic AR CC-4 from Mallinckrodt, St. Louis, MO. Aquacide III was from Calbiochem, CA and Ultrogel AcA 54 from LKB, France. The sources of the materials used in the enzyme-linked immunosorbent assay (ELISA) are described elsewhere [29]. Pig pancreatic phospholipase $A_2$ and phosphatidylcholine exchange protein from bovine liver were kindly donated by Dr. A.J. Slotboom and Dr. K.W.A. Wirtz, respectively, from this laboratory.

*Substrates.* 1-Acyl-2-[1-$^{14}$C]linoleoyl-*sn*-glycero-3-phosphocholine and -ethanolamine were biochemically prepared [30]. 1-*O*-Hexadecyl-2-[9,10-$^3H_2$]oleoyl-*sn*-glycero-3-phosphocholine was chemically prepared by acylation of 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine with [9,10-$^3H_2$]oleic acid anhydride in dry chloroform in the

presence of *N,N*-dimethyl-4-aminopyridine [31,32]. The products were extracted from the reaction mixtures [33] and purified by silica gel column chromatography followed by one-dimensional preparative thin-layer chromatography on silica gel 60 HPTLC plates (Merck, F.R.G.). The plates were developed first with petroleum ether (40–60 °C)/diethyl ether/formic acid (120 : 80 : 3, v/v) to remove traces of free fatty acids and then with chloroform/methanol/water/conc. ammonia (68 : 28 : 2 : 2, v/v) to separate phospholipids. At least 97% of the radiolabeled fatty acids could be removed from the substrates upon incubation with porcine pancreatic phospholipase $A_2$.

*Preparation of tissue cytosol and plasma.* Tissues of adult Wistar rats (150–200 g) were excised after decapitation and washed in 150 mM KCl/50 mM Tris/10 mM β-mercaptoethanol/3 mM $MgCl_2$/0.1 mM EGTA (buffer A, pH 7.4). A 20% (w/v) homogenate in buffer A was prepared with a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 10 min at 600 × *g* and 20 min at 20 000 × *g*. The resulting supernatants were used to prepare microsomal and cytosol fractions after 60 min centrifugation at 105 000 × *g*. The microsomal pellet was resuspended in buffer A to a concentration of 5 mg protein per ml. In some experiments, the rats were anaesthesized with pentobarbital, injected with heparin and perfused with saline to obtain essentially blood-free lungs, which where processed as described above.

Freshly obtained citrated rat blood (3 parts blood collected in 1 part citrate-dextrose solution) was centrifuged for 10 min at 2500 × *g* and then for 60 min at 105 000 × *g* to yield clear plasma.

*Labeling of lung microsomal membranes with radioactive phosphatidylcholines.* Microsomes were labeled by incubation with vesicles of radioactive phosphatidylcholines in the presence of phosphatidylcholine exchange protein as described [34]. Briefly, mixtures of 50 nmol 1-acyl-2-[9,10-$^3H_2$] oleoylphosphatidylcholine (40 000 dpm/nmol) and 50 nmol of either 1-acyl-2-[1-$^{14}$C]palmitoyl-, 1-acyl-2-[1-$^{14}$C]linoleoyl- or 1-acyl-2-[1-$^{14}$C]arachidonoylphosphatidylcholine (each 20 000 dpm/nmol) in the presence of 2 nmol phosphatidic acid were sonicated in 3 ml of buffer A with a Branson B12 sonifier for 45 min at 50 W under $N_2$ at 0 °C. The sonicate was centrifuged for 60 min at 105 000 × *g*. The supernatant was mixed with 3 ml of resuspended lung microsomes (15 mg protein) and incubated for 45 min at 37 °C in the presence of 72 μg phosphatidylcholine exchange protein. After the incubation, microsomes were separated from vesicles by centrifugation at 105 000 × *g* for 60 min and resuspended in buffer A to a concentration of 5 mg/ml.

*Phospholipase $A_2$ assyas.* The labeled substrates were sonicated four times for 15 s in 50 mM Tris-maleic acid/1 mM EGTA buffer (pH 6.5) at a concentration of 0.5 μmol/ml. Sonicated substrate (0.1 ml) was incubated for 60 min at 37 °C with varying amounts of cytosol (0.1–1 mg protein) after adjusting the total volume to 0.2 ml with buffer A. When $Ca^{2+}$-dependent phospholipase $A_2$ was to be determined, 1.7 μmol $Ca^{2+}$ (final conc. 8 mM) was included in the incubation mixtures. Hydrolysis of microsomal-associated phosphatidylcholines was measured for 30 min at 37 °C in incubations containing variable amounts of labeled microsomes and 0.35 ml of lung cytosol in a total volume of 0.5 ml. Released labeled fatty acids were determined after a Dole extraction procedure followed by mini-column silica gel chromatography as described by Van den Bosch et al. [30]. In control experiments, the radioactive products were applied to HPTLC silica gel 60 plates. After development with petroleum ether (40–60 °C)/diethyl ether/formic acid (120 : 80 : 3, v/v), staining with iodine and destaining with $SO_2$, the regions corresponding to 1,2- and 1,3-diradylglycerol ($R_F$ = 0.26–0.30) and free fatty acid ($R_F$ = 0.47) were scraped and transferred into toluene-based scintillation fluid for radioactivity measurement.

*Gel filtration of lung cytosol.* Lung cytosol isolated from 20% (w/v) homogenates from ten rat lungs was adjusted to pH 5.1 by addition of 0.5 M acetic acid. After gentle stirring for 10 min at 0 °C, the mixture was centrifuged for 10 min at 27 000 × *g*, and the supernatant brought to pH 7.4 with 0.5 M NaOH and concentrated by dialysis against Aquacide III. After dialysis of the concentrated delipidated lung cytosol against buffer A supplemented with 1 M KCl/10% (v/v) glycerol/0.02% (w/v) $NaN_3$, the solution was applied to an Ultrogel AcA 54 column (96 × 2.2 cm)

equilibrated with the dialysis buffer.

*Analytical procedures.* Fractions of the AcA 54 gel filtration were analyzed for immunochemical cross-reactivity with monoclonal antibody 2B9B7C5, sub-cloned twice from hybridoma 2B9, against rat liver mitochondrial phospholipase $A_2$ as described [29]. Protein was determined with the Bio-Rad protein assay according to Bradford [35], with bovine serum albumin as standard. Phospholipid phosphorus in lipid extracts [33] from cytosols was determined after ashing [36]. Calcium contents of cytosols were analyzed with a Varian-Techtron 1200 atomic absorption spectrometer operating with an air/acetylene flame by measuring the absorbance at 423 nm of cytosols and 0–100 $\mu$M $CaCl_2$ standards in 0.15 M KCl.

## Results and Discussion

### *$Ca^{2+}$-independent phospholipase $A_2$ activity in rat tissue cytosols*

Cytosols of seven rat tissues (Fig. 1) were screened for the presence of $Ca^{2+}$-independent phospholipase $A_2$ activity by measuring the release of radioactive fatty acids from three substrates, i.e, 1-acyl-2-[1-$^{14}$C]linoleoyl-*sn*-glycero-3-phosphoethanolamine, 1-acyl-2-[1-$^{14}$C]linoleoyl-*sn*-glycero-3-phosphocholine and 1-*O*-hexadecyl-2-[9,10-$^{3}H_2$]oleoyl-*sn*-glycero-3-phosphocholine in the presence of excess EGTA. Rat blood plasma was also tested, but its activity was below the detection limit of 1 pmol · min$^{-1}$. Fig. 1 summarizes the results obtained with tissue cytosols based on assays that were linear with time and protein. Highest activities were found in spleen, liver, lung and brain, especially with phosphatidylethanolamine as substrate. In spleen, liver and lung, this substrate gave about 6-fold higher hy-

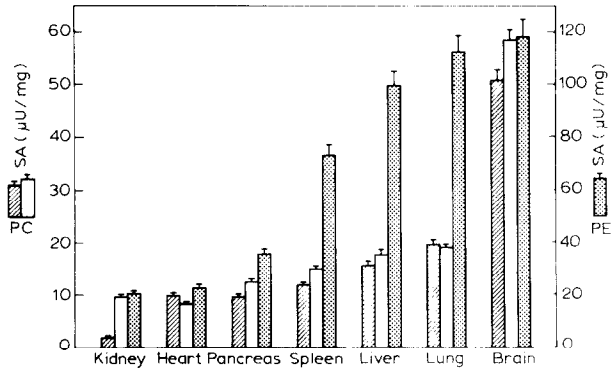


Fig. 1. $Ca^{2+}$-independent phospholipase $A_2$ activity of rat tissue cytosols. Phospholipase $A_2$ activity, determined as described in Materials and Methods, is expressed as specific activity (SA) in pmol labeled fatty acid released/min per mg cytosol protein ($\mu$U/mg) from phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Hatched bars indicate 1-*O*-hexadecyl-2-[9,10-$^{3}H_2$]oleoyl-*sn*-glycero-3-phosphocholine (spec. act. 2030 dpm/nmol), open bars 1-acyl-2-[1-$^{14}$C]linoleoyl-*sn*-glycero-3-phosphocholine (spec. act. 2620 dpm/nmol) and dotted bars 1-acyl-2-[1-$^{14}$C]linoleoyl-*sn*-glycero-3-phosphoethanolamine (spec. act. 2500 dpm/nmol) as substrate. Values represent means of duplicate determinations each from two separate cytosol isolations after correction for blank incubations without cytosol. Standard errors of the means are indicated on top of the bars.

TABLE I

ANALYSES OF RAT TISSUE CYTOSOLS

Tissue cytosols from two rats were isolated separately and analyzed as described in Materials and Methods. Mean values, differing by less than 10%, are indicated. P-lipid, phospholipid.

| Tissue | Wet weight (g) | Cytosol | | | | $Ca^{2+}$-independent phospholipase $A_2$ [a] | | |
|---|---|---|---|---|---|---|---|---|
| | | vol. (ml) | protein (mg · ml$^{-1}$) | P-lipid (nmol · mg$^{-1}$) | $Ca^{2+}$ ($\mu$M) | spec. act. (pmol · min$^{-1}$ · mg$^{-1}$) | total (mU) | content (mU/g wet weight) |
| Kidney | 1.4 | 7.1 | 9.5 | 18 | 42 | 1.7 | 0.11 | 0.08 |
| Heart | 0.6 | 3.0 | 8.3 | 4 | 38 | 11.6 | 0.29 | 0.42 |
| Pancreas | 0.5 | 2.5 | 10.1 | 3 | 85 | 10.1 | 0.26 | 0.52 |
| Spleen | 0.5 | 2.5 | 10.0 | 6 | 26 | 13.1 | 0.33 | 0.66 |
| Liver | 8.7 | 43.3 | 16.7 | 3 | 36 | 16.4 | 11.86 | 1.36 |
| Lung | 1.0 | 5.0 | 9.7 | 11 | 41 | 20.3 | 0.98 | 0.98 |
| Brain | 1.8 | 9.0 | 5.0 | 16 | 37 | 49.5 | 2.23 | 1.24 |

[a] Phospholipase $A_2$ activity was assayed with 1-*O*-hexadecyl-2-[9,10-$^{3}H_2$]oleoyl-*sn*-glycero-3-phosphocholine to avoid interference by consecutive phospholipase $A_2$ and lysophospholipase action.

drolysis rates than observed for phosphatidylcholines (note scale difference). This preference is somewhat more pronounced than the 2- to 3-fold higher activity on phosphatidylethanolamine noted previously for lung and liver homogenates [37]. Kidney, heart, pancreas and brain cytosols hydrolyzed phosphatidylethanolamine about twice as fast as phosphatidylcholine. These differences most likely reflect properties of the enzymes, and do not appear to be caused by different dilutions of the exogenous substrates by endogenous phospholipids present in the cytosol preparations. Although the phospholipid content per mg protein varied considerably for the different cytosols (Table I), the amount of cytosolic phospholipid added to the incubations never exceeded 10 nmol, and hence rendered dilution of the 50 nmol substrate rather unimportant. With the exception of kidney, all cytosols degraded 1-*O*-hexadecyl-2-[9,10-$^3H_2$]-oleoyl-*sn*-glycero-3-phosphocholine and 1-acyl-2-[1-$^{14}$C]linoleoylphosphatidylcholine equally well. This finding strongly suggests that a consecutive action of phospholipase $A_1$ and lysophospholipases did not contribute significantly to the release of *sn*-2-position fatty acids from phosphatidylcholine. However, this conclusion cannot be drawn for phosphatidylethanolamine hydrolysis in that this substrate contains a phospholipase $A_1$-susceptible 1-acyl ester bond. Some of the differences in relative hydrolysis rates for phosphatidylcholine and phosphatidylethanolamine (Fig. 1) may therefore be explained by the additional presence of phospholipases $A_1$ with preference for phosphatidylethanolamine and lysophospholipases in the cytosol fractions. The presence of these enzymes in rat liver cytosol has been documented before [38,39]. In view of these considerations, the data obtained with 1-*O*-hexadecyl-2-[9,10-$^3H_2$]oleoyl-*sn*-glycero-3-phosphocholine were considered to give the most reliable indications for phospholipase $A_2$ activity. Control experiments with all cytosols and all three substrates utilized indicated that always over 85% of the released radioactivity was associated with free fatty acid and this did not change with incubation time, indicating phospholipase $A_2$ activity. Since the incubations contained 110 nmol EGTA and the maximal amounts of $Ca^{2+}$, which were added by using 1 mg of cytosolic protein could have amounted to 2.2–8.5 nmol $Ca^{2+}$ (liver and pancreas, respectively (Table I)) these phospholipase $A_2$ activities were clearly $Ca^{2+}$-independent. Total levels of cytosolic $Ca^{2+}$-independent phospholipase $A_2$ activity in tissues and activities per gram tissue are presented in Table I. With the exception of kidney, the activities per g tissue are strikingly constant, ranging from 0.4 mU/g for heart to 1.4 mU/g for liver. The rather uniform occurrence of $Ca^{2+}$-independent phospholipase $A_2$ in rat tissues perhaps suggests a general function in phospholipid turnover and for acyl chain remodeling processes under the intracellular conditions of sub-micromolar $Ca^{2+}$-concentrations.

*Hydrolysis of microsomal-associated phosphatidylcholines by rat lung cytosolic $Ca^{2+}$-independent phospholipase $A_2$*

After demonstration of the presence of $Ca^{2+}$-independent phospholipase $A_2$ activity in rat tissue cytosols, we addressed the question of whether such soluble enzymes were capable of attacking membrane-associated substrates. These experiments were performed with rat lung microsomes and lung cytosol in view of the proposed involvement of this enzyme in cytosol-stimulated $Ca^{2+}$-independent remodeling of phosphatidylcholine in rat lung microsomes [40]. For this purpose, traces of the endogenous phosphatidylcholines of isolated lung microsomes were exchanged for phosphatidylcholine species containing either labeled palmitoyl, oleoyl, linoleoyl or arachidonoyl moieties esterified at the *sn*-2-position (Table II). In as much as sonicated phosphatidylcholine vesicles contain about 65% of their constituent lipids in the outer monolayer [41] and only these are accessible for exchange [42], it can be concluded from the amount of vesicular [$^3$H]phosphatidylcholine recovered in the microsomes that the exchange process had reached near equilibrium. This incorporation of about 50 nmol labeled phosphatidylcholine in microsomal membranes, equivalent to 15 mg protein, and hence about 6000 nmol microsomal phosphatidylcholine, indicates that about 1% of the endogenous phosphatidylcholine had been replaced by the indicated species, and it can safely be assumed that the physical membrane properties did not change grossly by the introduction of these species. Fig. 2 shows the activity of

TABLE II

LABELING OF RAT LUNG MICROSOMES WITH PHOSPHATIDYLCHOLINE SPECIES BY EXCHANGE

Microsomes were labeled by phosphatidylcholine (PC) exchange, as described in Materials and Methods.

| PC species introduced [a] | Incorporation [b] (%) | Specific radioactivity [c] of microsomal PC (dpm/nmol) | |
|---|---|---|---|
| | | $^3H$ | $^{14}C$ |
| [$^3$H]Oleate + [$^{14}$C]palmitate | 55 | 201 | 99 |
| [$^3$H]Oleate + [$^{14}$C]linoleate | 61 | 183 | 124 |
| [$^3$H]Oleate + [$^{14}$C]arachidonate | 56 | 194 | 110 |

[a] Species are denoted by their labeled fatty acid at the *sn*-2 position.

[b] Calculated for [$^3$H]oleate species.

[c] Specific radioactivities of the total microsomal PC pool were calculated under the assumption that the labeled PC was distributed homogeneously among total microsomal PC.

lung cytosolic phospholipase $A_2$ towards microsomal-associated phosphatidylcholines in the presence of EGTA. The results indicate that lung cytosol is capable of releasing fatty acids from the *sn*-2-position of phosphatidylcholine in the absence of free $Ca^{2+}$. Thus, the soluble $Ca^{2+}$-independent phospholipase $A_2$ can act on membrane-associated substrates. In this action, the enzyme did not exhibit a strong preference for any of the species investigated, although the release of fatty acid from 1-acyl-2-[1-$^{14}$C]palmitoyl-*sn*-glycero-3-phosphocholine seems to be somewhat lower than that for phosphatidylcholine species with an unsaturated fatty acid at the *sn*-2-position. These data further support the idea that the $Ca^{2+}$-independent phospholipase $A_2$ in rat lung cytosol might contribute to the cytosol-stimulated remodeling of unsaturated phosphatidylcholines in rat lung microsomes noted previously [40]. A somewhat lower hydrolysis of palmitate from the *sn*-2-position of phosphatidylcholine in combination with a reacylation of the lysophosphatidylcholines produced from unsaturated phosphatidylcholines by the abundant palmitoyl-CoA in rat lung cytosol [40] could explain the preferential formation of surfactant dipalmitoylphosphatidylcholine by deacylation-reacylation processes operating under conditions of low intracellular $Ca^{2+}$ concentrations. However, it should be realized that surfactant dipalmitoylphosphatidylcholine is synthesized in type II cells and that the cellular origin of lung cytosolic $Ca^{2+}$-independent phospholipase $A_2$ remains unknown at present. Rat lung contains, per g wet weight, approx. 10 μmol phosphatidylcholine [43] and 50 mg cytosolic protein (Table I). Assuming that substrate and $Ca^{2+}$-independent phospholipase $A_2$ are equally distributed throughout the tissue and that the enzyme operates under saturating conditions of membrane-associated substrate, an activity of 2 nmol · $h^{-1}$ · $mg^{-1}$ (Fig. 2) could degrade half of the total lung phosphatidylcholine pool in about 50 h.

*Assay for immunochemical cross-reactivity of rat lung cytosol phospholipase $A_2$ with monoclonal antibodies against rat liver mitochondrial phospholipase $A_2$*

Previous experiments have shown that rat lung cytosol contained both $Ca^{2+}$-dependent and

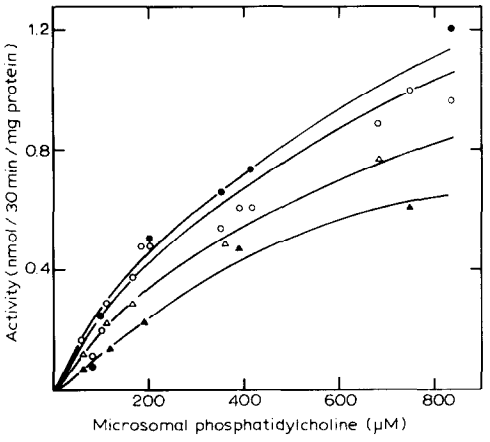


Fig. 2. $Ca^{2+}$-independent phospholipase $A_2$ activity from rat lung cytosol on microsomal-associated phosphatidylcholine. Cytosol and labeled microsomes were prepared as described in Materials and Methods. Incubations contained 0.35 ml cytosol (3.4 mg protein) and varying amounts of labeled rat lung microsomes equivalent to the indicated amounts of phosphatidylcholine in a total volume of 0.5 ml buffer A. The release of radioactive fatty acid is given for microsomes labeled with 1-acyl-2-[9,10-$^3H_2$]oleoylphosphatidylcholine (○) and either 1-acyl-2-[1-$^{14}$C]palmitoyl- (▲), 1-acyl-2-[1-$^{14}$C]linoleoyl- (●) or 1-acyl-2-[1-$^{14}$C]arachidonoylphosphatidylcholine (△). Values are corrected for blank incubations without cytosol and are normalized to oleate release from the individual microsomal preparations.

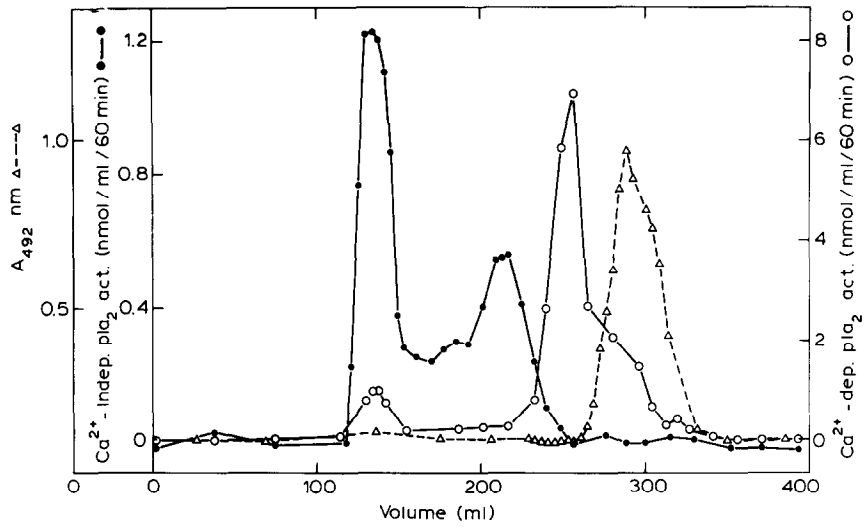


Fig. 3. Gel filtration of rat lung cytosol. An aliquot of delipidated and concentrated rat lung cytosol (3 ml, 40 mg protein, 0.96 mU $Ca^{2+}$-independent phospholipase $A_2$) was filtered over Ultrogel AcA 54 (96×2.2 cm) at a flow rate of 24 ml/h. Fractions of 4 ml were collected. Aliquots of 100 μl were assayed for phospholipase $A_2$ activities, both in the absence and presence of $Ca^{2+}$, with 1-acyl-2-[1-$^{14}$C]linoleoylphosphatidylcholine as substrate. $A_{492\,nm}$ represents absorbance measured in ELISA reactions with 50 μl column fractions and monoclonal antibodies against rat liver mitochondrial $Ca^{2+}$-dependent phospholipase $A_2$. Recoveries of protein (not shown) and $Ca^{2+}$-independent phospholipase $A_2$ activity amounted to 90 and 70%, respectively.

$Ca^{2+}$-independent phospholipase $A_2$ activities [24]. These could be separated by gel filtration. Thus, cross-reactivity of each of these phospholipase $A_2$ activities with monoclonal antibodies against an intracellular phospholipase $A_2$ from rat liver [29] can conveniently be studied after gel filtration of lung cytosol. Since lung tissue, as normally isolated, contains some blood, and rat platelets contain a $Ca^{2+}$-dependent phospholipase $A_2$ which is cross-reactive with the above-men-

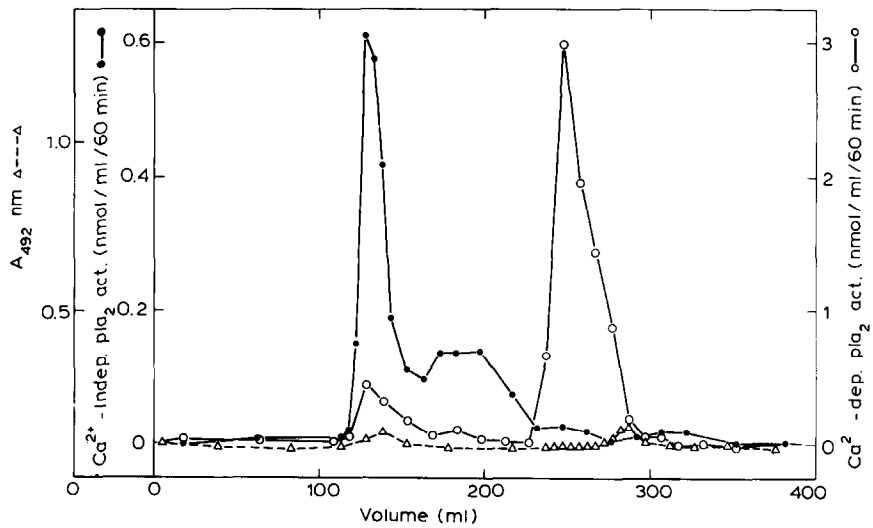


Fig. 4. Gel filtration of cytosol prepared from perfused rat lung. Conditions and assays were as described in the legend of Fig. 3, except that the column was loaded with delipidated and concentrated cytosol (1.6 ml, 21.3 mg protein, 0.64 mU $Ca^{2+}$-independent phospholipase $A_2$) prepared from perfused rat lungs. Recoveries of protein (not shown) and $Ca^{2+}$-independent phospholipase $A_2$ activity amounted to 78 and 58%, respectively.

tioned monoclonal antibodies, these experiments were also performed with cytosol prepared from perfused rat lung. Fractions of the gel filtration columns were assayed with 1-acyl-2-[1-$^{14}$C]linoleoyl-*sn*-glycero-3-phosphocholine in the presence of EGTA for $Ca^{2+}$-independent phospholipase $A_2$ and in the presence of excess $Ca^{2+}$ for $Ca^{2+}$-dependent phospholipase $A_2$ activity. Together with the absorbances at 492 nm obtained in ELISA experiments, both phospholipase $A_2$ activities are depicted in Figs. 3 and 4 for cytosol isolated from normal and perfused rat lung, respectively. All peak fractions were also assayed for phospholipase $A_2$ activity with 1-*O*-hexadecyl-2-[9,10-$^3H_2$]oleoyl-*sn*-glycero-3-phosphocholine. Activities were comparable with those obtained with diacylphosphatidylcholine, and product identification showed that neither substrate was degraded by phospholipase C-type activities to a substantial extent (data not shown). Detectable protein eluted from both columns in the eluate from 115 to 240 ml, with two main peaks around 135 and 190 ml. The elution profile of cytosol prepared from non-perfused lungs (Fig. 3) showed the presence of at least three phospholipase $A_2$ activities. Two peaks, eluting at 130 and 210 ml were detected in the presence of EGTA. The first of these gave comparable activity in assays in the presence of excess $Ca^{2+}$ (note scale difference). The second peak, preceded by a shoulder, was only observed when assayed in the presence of EGTA, and apparently is inhibited in the presence of 8 mM $Ca^{2+}$. Both peaks were also detected when phosphatidylethanolamine was used as substrate (data not shown), and showed then identical behaviour with respect to EGTA and $Ca^{2+}$ effects, as found for phosphatidylcholine. Interestingly, this second peak of $Ca^{2+}$-independent phospholipase $A_2$ activity which eluted at 210 ml, was no longer observed in cytosol from perfused lungs (Fig. 4), strongly suggesting that it originated from blood cells. The shoulder eluting at 185 ml upon gel filtration of cytosol from non-perfused lungs (Fig. 3) now became visible as a broad peak (Fig. 4) and apparently, like the peak eluting at 130 ml, is derived from lung tissue.

The relative proportion of the activities eluting at 130 and 185 ml is similar in cytosols from non-perfused and perfused lungs. Yet, it cannot be concluded whether these peaks represent different $Ca^{2+}$-independent phospholipases $A_2$ or whether they are caused by a single enzyme in free and lipid- or protein-associated form. None of these $Ca^{2+}$-independent phospholipase $A_2$ activities, including the one derived from blood cells, showed any cross-reactivity with monoclonal antibodies directed against rat liver mitochondrial $Ca^{2+}$-dependent phospholipase $A_2$. Cross-reactivity was observed, however, with the $Ca^{2+}$-dependent phospholipase $A_2$ activity present in cytosol from non-perfused lungs, but only with the tail part of the enzymatic activity peak (Fig. 3). This cross-reactive material has almost completely disappeared is cytosol from perfused lungs (Fig. 4), and most likely represents platelet phospholipase $A_2$. It was recently shown that this enzyme is released from rat platelets during activation [44,45] and is cross-reactive with monoclonal antibodies against rat liver mitochondrial phospholipase $A_2$ [29]. However, the main peak of $Ca^{2+}$-dependent phospholipase $A_2$ eluting at 250 ml remained present in cytosol from perfused lungs in a fairly constant proportion with that of the $Ca^{2+}$-independent phospholipase $A_2$ activity, indicating that it is also derived from lung tissue. It is interesting to note that this enzyme with a similar $Ca^{2+}$ requirement and a similar, albeit perhaps slightly higher (Fig. 3), molecular weight did not cross-react with monoclonal antibodies that recognize rat platelet and rat liver mitochondrial phospholipase $A_2$. This further emphasizes the heterogeneity of $Ca^{2+}$-dependent cellular phospholipase $A_2$.

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