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Phospholipase D in Guinea Pig Lung Tissue Membrane is Regulated by Cytosolic ARF Proteins

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Abstract Phospholipase D (PLD) and ADP-ribosylation factor (ARF) were partially purified on a series of column chromatography, and their biochemical properties were characterized to understand the regulatory mechanism of PLD activation by ARF protein in the antigen-induced immune responses in guinea pigs. Heparin Sepharose and high-Q Sepharose column chromatographies were used for the purification of PLD, and Sephadex G-25, DEAE Sephacel, Source 15 PHE (HIC), Superdex-75, and Uno-Q column chromatographies were used for the purification of ARF. The purified PLD and ARF proteins were identified with anti-rabbit PLD- or ARF-specific antibodies, showing about 64 or 85 kDa for the molecular mass of PLD and 29 or 35 kDa for the sizes of ARF. Partial cDNA of ARF3 was cloned by RT-PCR in guinea pig lung tissue and its nucleotides and amino acids were sequenced. Guinea pig ARF3 showed 92% of nucleotides sequence identity and 100% of amino acid sequence homology with human ARF3. The ARF-regulated PLD activity was measured in the oleate or ARFs-containing mixed lipid vesicles. The purified and recombinant ARF (rARF) activities were assessed with the GTPγS binding assay. The PLD activity was induced by oleate in a dose-dependent manner. The purified ARF and recombinant ARF3 increased PLD activity in guinea pig lung tissues. These data show that the activity of membrane-bound PLD can be regulated by the cytosolic ARF proteins, suggesting that ARF proteins in guinea pig lung can act as a regulatory factor in controlling the PLD activity in allergic reaction.

Key words: Phospholipase D (PLD), ADP-ribosylation factor (ARF), guinea pig lung tissues

The last few decades have seen the emergence of a large number of new lipids and lipid-derived messengers. These messengers are produced by a number of signal-activated phospholipase enzymes and lipid kinases. One of these enzymes is phospholipase D (PLD), a phosphodiesterase that is increasingly recognized as an important signal-transducing enzyme. The PLD catalyzes the hydrolysis of phosphatidylcholine (PC) to generate phosphatidic acid (PA) and choline [17]. The PLD activity in mammalian cells and tissues is low and transiently increases following occupation of many cell surface receptors, including those of the heterotrimeric G-protein-coupled receptors and tyrosine kinase receptors [9, 15, 19].

Biochemical evidence suggests that there are multiple PLD isozymes (hPLD1 and hPLD2) in mammalian cells [10, 20] including RBL 2H3 mast cells [11] and rat neutrophils [8]. A membrane-bound PLD seems to be an integral membrane protein that is highly specific for PC as a substrate and is activated by sodium oleate. A cytosolic PLD is less strict in its substrate specificity, because it can hydrolyze phosphatidylethanolamine, PC, and phosphatidylinositol (PI). Furthermore, the cytosolic PLD activity in the rat pancreatic acinar cells and rat brain cytosol remains poorly characterized [3, 32].

A membrane-bound PLD that behaves as a peripheral membrane protein has been found in the various cell types [5, 18, 22, 24, 40, 43]. The membrane PLD is regulated by many factors including protein kinase C (PKC), phosphatidylinositol 4,5-bisphosphate (PIP_2), the small G-proteins such as ADP-ribosylation factor (ARF) [11], and the Rho families [5]. Recently, studies on the regulation of PLD1 have increased greatly due to the cloning of several isoforms of the mammalian PLD [8, 12, 27-28, 33-34]. Therefore, it seems highly likely that multiple PLD isozymes are involved in diverse functions through the regulation of their activities.

In contrast to the finding with hPLD1, hPLD2 exhibits high basal activity in the presence of PIP2. It does not respond to Rho A or PKC, and is weakly stimulated by

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ARFs [13, 33]. It is activated by ARF6 in intact RBL 2H3 mast cells [11], and can be activated by unsaturated fatty acid, including oleate, linoleate and arachidonate [29]. Based on the different biochemical and regulatory properties of PLD in many tissues [13-14, 29, 32-34], it seems likely that certain isoforms of this enzyme, which are more selective in their regulation by ARF, Rho A, and PKC, should exist and will be identified.

ARF (~21 kDa), a member of the monomeric GTP-binding protein family was first identified and purified as a cofactor for cholera toxin-catalyzed ADP-ribosylation of heterotrimeric GTP-binding proteins [2, 4, 7, 27]. Similar to other G-proteins, ARFs cycle between inactive GDP-bound and active GTP-bound conformations, and they seem to shuttle between the cytosol and the membrane during the activation cycle [4]. There are six ARF proteins, ARF1-ARF6, and all of them activate PLD1 in most cell types, including mast cells [8, 11, 19, 22, 40, 43]. On the other hand, only ARF1 or ARF6 among the ARFs activates PLD2 in some cell types [11].

It is already well known that mast cells play a pivotal role in the pathogenesis of allergic reactions such as asthma. The multivalent binding of antigen to FcεRI receptor-bound IgE triggers the release of inflammatory mediators through the exocytosis of secretary granules, the generation of arachidonic acid metabolites, and the production of cytokines [1, 35, 37]. We previously reported that the PLD, PKC, PLA_2, and Ca^{2+} are dominantly activated in guinea pig lung and human cord blood-derived mast cells, when stimulated with antigen/antibody reactions [38-39].

The allergic reactions and responses observed in both guinea pigs and humans share very similar characteristics with many other species. As described above, the PLD is activated by ARFs in rat brain, pig brain, and liver, as well as cell lines including guinea pig lung mast cells. However, there are no reports on the PLD isolated or identified from guinea pig lung tissues, an important organ with regard to asthma, and no reports on the regulation of PLD by ARF in the organ. Therefore, in the present study, we attempted to confirm whether PLD exists in guinea pig lung tissues and whether it is regulated by a small G-protein, ARFs, and to show whether or not there are any species differences in its biochemical characteristics.

MATERIALS AND METHODS

Materials

Phosphatidylcholine, L-α-dipalmitoyl, [choline-methyl-^{3}H] ([^{3}H]PtdCho. 30-60 Ci/mmol) and guanosine 5'-(γ-thio) triphosphate ([^{35}S]GTPγS. 1,250 Ci/mmol) were purchased from New England Nuclear (NEN, Seoul Korea) Life Science Products. PMSF, dithiolthreitol (DTT) were obtained from DUCHEFA (Amsterdam, The Netherlands). Aprotinin, leupeptin, sodium salts of oleic acid, L-α-phosphatidyl-ethanolamine, L-α-phosphatidylinositol 4,5-diphosphate, L-α-phosphatidylcholine, di-palmitoyl (C16: 6), adenosine 5-triphosphate (ATP), guanosine 5'-[γ-thio] triphosphate (GTPγS), and n-octyl-β-D-glucopyranoside were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). Rabbit anti-PLD C-terminal antibody and antibody against human neutrophil-derived recombinant ARF (rARF) were kindly provided by Dr S. H. Ryu (Phohang University of Science and Technology, Phohang, Korea). ARF antibody and secondary antibody (Conjugated HRP anti-rabbit IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Hybond-C EXTRA nitrocellulose (0.45 μm pore size) and Glutathione Sepharose were from Amersham Pharmacia Biotech (Uppsala, Sweden). Trizol solution was from Invitrogen (Groningen, The Netherlands), and RT-PCR kit from GIBCO-RRL (Grand Island, NY, U.S.A.). All other chemicals were of the highest grade available.

Preparation of Membrane from Guinea Pig Lung Tissues

All procedures were carried out at 4°C unless otherwise indicated. Guinea pig lung (90 g) was homogenized for 2 min at 9,500 rpm by Polytron homogenizer in 7 volumes (630 ml, w/v) of buffer A [20 mM Na-Hepes (pH 7.4), 1 mM EDTA, 0.1 mM dithiolthreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and leupeptin and aprotinin (each at 1.5 μg/ml)]. The homogenate was centrifuged at 1,000 ×g for 20 min. The pellet was further homogenized in 2 volumes of buffer A with Polytron homogenizer. The homogenate was centrifuged at 10,000 ×g for 20 min. Supernatants were pooled and then centrifuged at 100,000 ×g for 1 h. The supernatant from the two homogenization steps were saved for the purification of ARF. The resulting membrane pellet was stirred for 1 h in the presence of 5 volumes (w/v) of buffer A containing 1% Triton X-100 and 0.3 M NaCl. Insoluble materials were discarded after centrifugation at 100,000 ×g for 1 h.

Partial Purification of Membrane-Bound PLD Enzyme in the Guinea Pig Lung Tissue

PLD was purified by the modified method described previously [21, 30]. Solubilized proteins were applied to a heparin-Sepharose CL-6B column (2.5×15 cm) that had been equilibrated with Buffer B [20 mM Na-Hepes (pH 7.4), 1 mM EGTA, 0.1 mM DTT, and 0.1% Triton X-100] containing 0.3 M NaCl. Unbound proteins were washed with buffer B containing 0.3 M NaCl. Bound proteins were eluted from the column with 400 ml linear gradient of 0.3 to 3.0 M NaCl in buffer B. Each fraction of 2 ml was collected and assayed for PLD activity in the presence of GTPγS and purified ARF from guinea pig lung tissues in the presence of sodium oleate.

The peak activity fractions were pooled and diluted with 9 volumes of buffer B, and loaded onto a High-Q

Sepharose anion column (10×60 mm, bio-rad) which had been equilibrated in buffer B. Proteins were eluted from the column at a flow rate of 0.5 ml/min with 100 ml linear gradient of 0 to 1.0 NaCl in buffer C [20 mM Na-Hepes (pH7.4), 1 mM EGTA, 0.1mM DTT, and 0.7% n-octyl-β-D-glucopyranoside]. The eluates from the gradients were collected in 2 ml each fractions and were analyzed for PLD activity.

Partial Purification of Cytosolic ARF in the Guinea Pig Lung Tissues

The supernatant obtained after isolation of membrane fraction from guinea pig lung tissues was gently stirred for 1 h by adding solid ammonium sulfate to yield a final saturation of 35%, and centrifuged at 10,000 ×g (Beckman, JA 10) for 30 min according to the modified method [31, 44]. The resulting supernatant was brought to 55% saturation with ammonium sulfate, stirred for 1 h, and centrifuged at 10,000 ×g for 30 min. The 35-55% ammonium sulfate precipitate was suspended in 50 ml of buffer B, applied to a Sephadex G-25 (5×100 cm) column that had been equilibrated with the same buffer, and the protein fractions containing ARF were pooled and insoluble particles were removed by filtration through glass fiber filter. The supernatant was loaded onto a DEAE-Sephadex column (5×20 cm) that had been equilibrated with buffer B. The column was washed with buffer B, and the bound proteins were eluted with a 1,600 ml of linear gradient of 0 to 0.5 M NaCl in buffer B. Peak fractions containing ARF activity were concentrated to 0.5 ml and applied to a Superdex-75 HR 10/30 gel filtration column (10×300 mm) that had been equilibrated with buffer D [20 mM Tris-Cl (pH 8.0), 1 mM EDTA, 100 mM NaCl, and 1 mM DTT]. Proteins were eluted at a flow rate of 0.25 ml/min, and peak fractions containing ARF were pooled and applied to a Uno-Q anion-exchange column that had been equilibrated with buffer E [20 mM Tris-Cl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT]. The column was washed with buffer E, and the bound proteins were eluted with 24 ml linear gradient of 0 to 1 M NaCl in buffer E. Proteins were eluted at a flow rate of 1 ml/min, and peak fractions were pooled and the purified ARF was confirmed with the anti-ARF specific antibody.

Measurement of Oleate-Dependent PLD Activity

Oleate-dependent PLD activity was measured in assay buffer containing 50 mM Hepes (pH 7.0), 2 mM EGTA, 4 mM sodium oleate, 500 mM KCl, 4 mM $MgCl_2$, and 0.1 mM free calcium. The reaction mixture which consisted of 25 µl of mixed lipid vesicles containing PtdEtn/$PtdInsIP_2$/$(Pam)_2$PtdCho(molar ratio 16:1.4:1) with [choline-methyl-^{3}H]$(Pam)_2$PtdCho to yield 200,000 cpm per assay, 20 µl of purified PLD, and 130 µl of assay buffer was incubated for 1 h at 30°C before the addition of 1 ml of stop solution containing $CHCl_3$/CH_3OH/concentrated HCl, 50:50:0.3 (v/v) and 0.3 ml of 0.1 M HCl/5 mM EGTA. After separation of the organic and aqueous phases by centrifugation, the radioactivity released in 0.5 ml of the aqueous phase was quantified by liquid scintillation spectrometry [14].

Measurement of ARF-Dependent PLD Activity

PLD activity was measured using the method described previously [7, 14] with a minor modification. Briefly, 25 µl of mixed lipid vesicles containing PtdEtn/$PtdInsIP_2$/$(Pam)_2$PtdCho(molar ratio 16:1.4:1) with [choline-methyl-^{3}H]$(Pam)_2$PtdCho to yield 200,000 cpm per assay were added to 20 µl of purified PLD, partially purified ARF (7.0, 14.0 ng) or recombinant ARF (human neutrophil derived rARF) and 10 µM GTPγS in a total volume of 125 µl containing 50 mM Hepes (pH 7.3), 3 mM EGTA, 80 mM KCl, 2.5 mM $MgCl_2$, and 300 nM of free calcium. Incubation was carried out at 37°C for 1 h before the addition of 1 ml of stop solution containing $CHCl_3$/CH_3OH/concentrated HCl, 50:30:0.3 (v/v) and 0.35 ml of 1 M HCl/ 5 mM EGTA. After separation of the organic and aqueous phases by centrifugation, the radioactivity released in 0.5 ml aqueous phases was quantified by liquid scintillation spectrometry.

Measurement of GTPγS-Dependent Partial Purified ARF Activity

The activity of the purified ARF was assessed by measuring its ability to associate with GTPγS by a method similar to those used for the assay of PLD. Partially purified ARF (7.0, 14.0 ng) was incubated with PtdEtn/$PtdInsIP_2$/$(Pam)_2$PtdCho lipid vesicles (as described above) in a reaction buffer, containing final concentrations of 20 mM Hepes (pH 7.4), 100 mM NaCl, 2 mM $MgCl_2$, 0.1% sodium cholate, 1 mM ATP, and [^{35}S]GTPγS (1 µM, 34 mCi/mol), for 60 min at 30°C. Reaction was quenched by the addition of 2 ml ice-cold stop buffer (reaction buffer containing 10 mM $MgCl_2$ and 100 mM GTPγS). GTP-bound ARF was separated through nitrocellulose filters (Whatman) and washed with ice-cold 20 mM Tris-Cl (pH 8.0), 100 mM NaCl, and 40 mM $MgCl_2$. The amount of [^{35}S]GTPγS bound was quantified by liquid scintillation counting [44].

Western Blot Analysis

Each purified fraction was analyzed with Western blot analysis. Proteins were separated by a denaturing SDS polyacrylamide gel and then transferred to a PVDF membrane. The membrane was then exposed sequentially to solutions containing primary antibody, followed by a secondary antibody to which an enzyme was coupled. The membrane was then soaked in a substrate solution to develop the color reaction, which resulted in a band. Apparent molecular weights of the protein were assessed using protein standard markers of known molecular weight.

RNA Isolation and 1st-Strand cDNA Synthesis

Total RNA from guinea pig lung tissue was isolated using Trizol, following the manufacturer's instructions (Invitrogen, Groningen, The Netherlands). cDNA was synthesized from 1.0 mg of the total RNA by Superscript™ 1st-Strand cDNA Synthesis System (Invitrogen, Groningen, The Netherlands).

PCR Cloning of ARF3 cDNA from Guinea Pig Lung Tissue

For the PCR cloning of ARF3 cDNA, the first strand of cDNA mixture was used as a template. Briefly, ARF3 DNA fragment (531 bp) was synthesized using PCR primers (ARF3-1N:5'-ATCATgggCAATATCTTTgg; ARF3-1 C: 5'-gAgCTGATTggCCAgCCAgT), corresponding to conserved sequences among human, rat, and mouse according to the modified method [26]. The optimized PCR reaction was performed for 35 cycles using thermal cycling parameters: 96°C for 30 sec followed by 53°C for 30 sec, and 72°C for 30 sec. An additional extension cycle was performed for 10 min at 72°C before cooling the reaction mixture to 4°C.

The resulting PCR product was cloned into the pGEM-T vector, referred to pGEM-ARF3, and the clone was transfected into *E. coli*. Six clones were isolated, and the plasmid DNA was extracted. DNA sequences were analyzed using the Taq dideoxyterminater cycle sequencing methods (Macrogen, Seoul, Korea), and consensus sequences were determined by comparing the sequences of the six clones.

Expression and Purification of the rARF3 Protein

Part of the open reading frame of ARF3 was amplified using pGEM-ARF3 as a template, a 5' sense primer with an *Eco*RI site (5'-gAgAgAgAgAggAATTCgg ATCATggg-CAATATCTTTgg), and a 3' reverse complement primer with a *Hin*dIII site (5'-gAgAgAgAgAgAgAAgCTTgAgCTgATT-ggCCAgCCAgT) for PCR products. Both the amplified product and the expression vector pGEX-4T2 were digested with *Eco*RI and *Hin*dIII, and then the PCR fragment was ligated to the pGEX-4T2. pGEX-4T2-transformed *E. coli* (*BL21* strain) was grown in LB medium with ampicillin. When the cell concentration reached to OD 1.0 at A_{600}, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM, and growth continued for an additional 2 h. The cells were collected by centrifugation, and suspended and sonicated in buffer containing 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, and 0.1% Triton-X 100. The lysate was centrifuged, the supernatant was applied to a Glutathione-Sepharose column, and then rARF3 was purified according to the manufacture's instructions using the Imidazole Gradient Method.

Statistical Analysis

Experimental data are shown as mean±S.E.M. An ANOVA was used for statistical analysis. An analysis of the significance between control and experimental groups was carried out with the Scheffes method. P values <0.05, 0.01, 0.001 were considered significant.

RESULTS

Purification and PLD Activity in Guinea Pig Lung Tissues

Guinea pig lung tissue membrane proteins were partially purified from the extractant by two column chromatographies. Two groups of fractions were obtained from Heparin Sepharose chromatography; the first fraction (fraction numbers 14-20) and the second fraction (fraction numbers 22-34) were based on the oleate-dependent activity assay (data not shown). Both pooled fractions of PLD activity, obtained from Heparin Sepharose, were applied to High-Q ion-exchange chromatography (Fig. 1a), and the column was eluted with salt gradient. One protein peak in the low salt range (15% NaCl) of High-Q Sepharose showed enzyme activity. However, the second protein fraction (fraction numbers 22-34) from the Heparin Sepharose column had

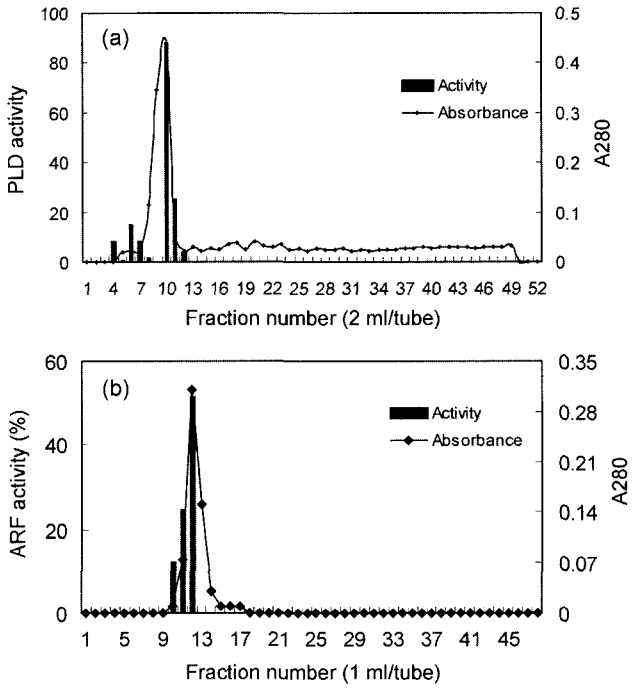

Fig. 1. Purification of PLD in membrane fraction and ARF in the cytosol fraction of guinea pig lung tissue.

(a) Purification of the PLD enzyme in membrane fraction isolated from guinea pig lung tissues was performed by Heparin Sepharose (data not shown) and High-Q ion-exchange chromatography. The oleate-dependent PLD activities were measured as described in Materials and Methods. (b) Purification of ARF was performed with various columns (data not shown) including Uno-Q column chromatography as described in Materials and Methods. The activities were accessed by quantification of GTPγS binding. Line indicates protein concentration and bar indicates relative activity. Activity (%), [(sample cpm-control cpm)]/control cpm×100.

Table 1. Summary of the purification procedure for membraneous PLD and cytosolic ARF isolated from guinea pig lung tissue[a].

Procedure	Total protein (mg[b])	(A-C)/C[c]	(A-C)/C/mg[d]	Fold	Recovery (%)
Membranes (PLD)	517.5	0.13	0.00025		100
High-Q	0.372	1.134	3.048	12,192	0.0007
35-55% cytosolic fraction (ARF)	148.65	0.47	0.0031		100
Uno-Q	0.0021	0.293	139.52	45,006	0.0014

[a]Purification of membrane-bound PLD and cytosolic ARF in the guinea pig lung tissues was performed by the methods described in Materials and Methods.
[b]The protein contents was measured with the Bradford method.
[c]The increased value after purification. A, sample values (cpm); C, control values (cpm).
[d]Sample values/mg of protein. High Q, High Q Sepharose column; Uno-Q, Uno-Q anion-exchange column.

two major protein peaks in a high salt range (55% NaCl), but neither protein peak showed PLD activity (data not shown). The activity and recovery for each fraction yielded from each column are summarized in Table 1.

Purification of ARF in Guinea Pig Lung Tissues

After being desalted with Sephadex G-25 column, 35-55% fraction protein was applied to various columns as described under Materials and Methods. High ARF activities were found in the fraction numbers of 46-51 in the DEAE Sephacel column. When the pooled fraction of DEAE column was applied to Superdex-75 gel filtration chromatography, two protein peaks were shown. Most of the activities in the two protein peaks were present in the second peak (fraction numbers 38-46). In the next step with Uno-Q column, the pooled fraction had activity with a very sharp peak and higher ARF (Fig. 1b). The activities and recovery for each fraction yielded from each column chromatography are summarized in Table 1.

Effects of Oleate and ARFs on the Partial Purified PLD Activity in the Guinea Pig Lung Tissues

It is known that PLD can be synergistically activated by ARF and oleate [14, 29]. To examine the effect of oleate on the PLD activity, the partially purified PLD were assayed in the presence of oleate. The PLD activities isolated from guinea pig lung tissues were strongly enhanced by oleate, showing a weak dose-dependency (Fig. 2a).

To examine the effects of partially purified ARF [and rARF (human neutrophil-derived recombinant ARF) on the partially purified PLD activity], the active fractions of ARF and rARF were added to the purified PLD (Fig. 2b). The increment in PLD activity in the presence of purified ARF was two-fold higher than that with rARF in each dose. However, the extent of PLD activation induced by rARF and purified ARF was less than that activated by oleate.

Confirmation of PLD and ARF Localized in the Guinea Pig Lung Tissues

To find the existence of PLD in the active fractions, immunoblotting analysis was carried out with rabbit anti-PLD antibody. Two bands were found in the active fraction (the pooled fraction numbers 9-11 eluted from High-Q column) (Fig. 3a). The molecular sizes of PLD from the guinea pig lung tissues were shown to be approximately 64 kDa and 85 kDa. However, it has been reported that the size of PLDs ranges from 90 to 120 kDa in various mammalian tissues. Therefore, our data suggest that the

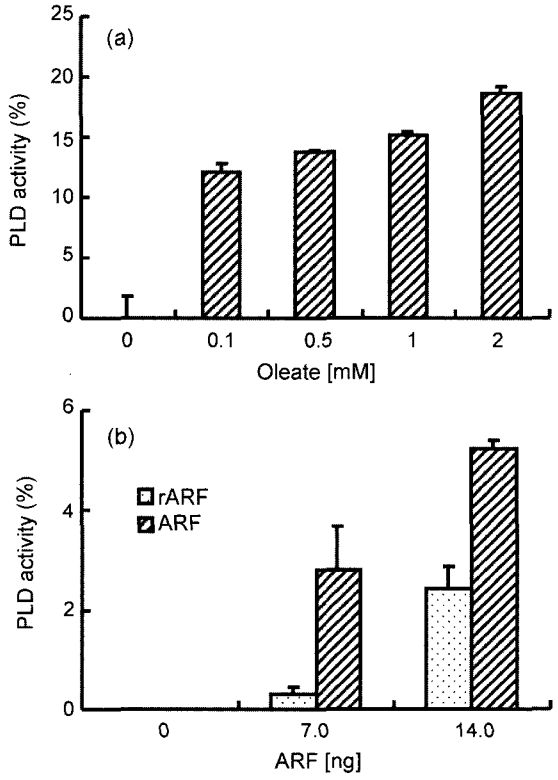

Fig. 2. Effects of oleate, rARF, and purified ARF on the guinea pig lung PLD activity.
The oleate and ARF(purified ARF and rARF)-induced PLD activities were assessed by measuring [^{3}H]choline released in aqueous phase. (a) The radioactivity of [^{3}H]choline released by PLD in the presence of oleate (0-2 mM) was measured as described in Materials and Methods. (b) Formation of [^{3}H]choline released by PLD in the presence of 10 mM GTPγS and rARF- or purified ARF was measured as described in Materials and Methods. Activity (%), [(sample cpm-control cpm)]/control cpm×100; rARF from human neutrophil-derived rARF.

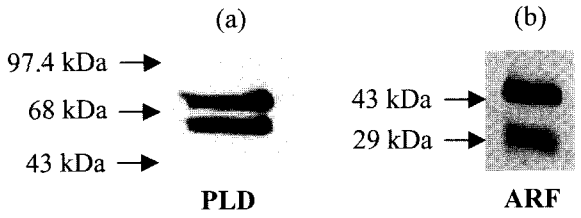

Fig. 3. Immunoblotting analysis of the purified PLD and ARF.
(a) PLD purified from the series of column chromatography and High-Q Sepharose ion-exchange chromatography was identified with rabbit anti-PLD specific antibody showing molecular mass of 85 and 64 kDa. (b) ARF isolated from Uno-Q ion-exchange chromatographies was detected with rabbit anti-ARF specific antibody, indicating molecular masses of 35 and 29 kDa.

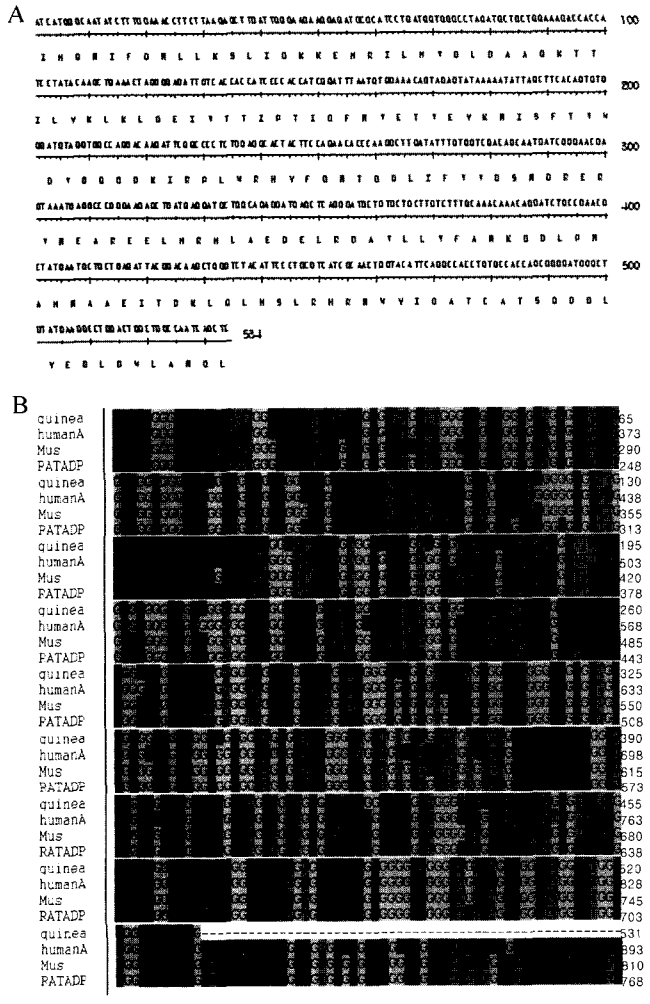

Fig. 4. Sequence analysis of partial cDNA of ARF3 isolated from the guinea pig lung tissue.
(A) Nucleotide sequence of partial cDNA of guinea pig ARF3 and its predicted amino acid sequence. (B) Comparison of ARF3 cDNA sequences from guinea pig (guinea), human (human A) (gil6995997l), mouse (Mus) (gil15928616l), and rat (RATADP) (gil438865l). Nucleotide sequences are numbered at the right side. A is red, T is green, C is blue, and G is yellow brown.

PLD isolated from guinea pig lung are different isoforms that may not exist in other mammalian tissues.

To find the existence of ARF in the active fractions, immunoblotting analysis was carried out with rabbit anti-ARF antibody. Two bands were found and their sizes were 29 and 43 kDa (Fig. 3b). Though the presence of ARF was shown by immunoblotting analysis in this study, the biochemical properties of ARF have not yet been completely characterized. They remain to be determined.

Nucleotide Sequencing Analysis of ARF3 in Guinea Pig Lung Tissues

The open reading frame (ORF) nucleotide of pGEX-ARF3 was sequenced in both strands. The nucleotide and amino acid sequences of guinea pig ARF3 are shown in Fig. 4. Sequence databases were searched using the Blast program (NCBI), and the translated ORF of the nucleotide identified a 177-amino acid protein with molecular mass of 20.1 kDa.

Nucleotide sequence similarity between human ARF3 and guinea pig ARF3 was about 92%, and the amino acid sequence of guinea pig ARF3 was found to have 100% identity with human ARF3 (3-177aa). The amino acid sequence of guinea pig ARF3 was 92% identical to the human ARF1 sequence.

Expression and Purification of the rARF3 Protein in Guinea Pig Lung Tissues

To assess any regulatory role of recombinant ARF3 (rARF3) cloned in guinea pig lung, GST-rARF3 was expressed in *E. coli* and purified by Glutathione-Sepharose column. The expression and purity of rARF3 were confirmed by SDS-PAGE, and the activity of rARF3-GST fusion protein was then assessed with GTP binding assay. The GTPγS binding activity of guinea pig rARF3 increased in a dose-dependent manner (from 37,721±3,304 cpm to 71,715±3,642 cpm for 9.0 mg rARF; to 105,295±11,090 cpm for

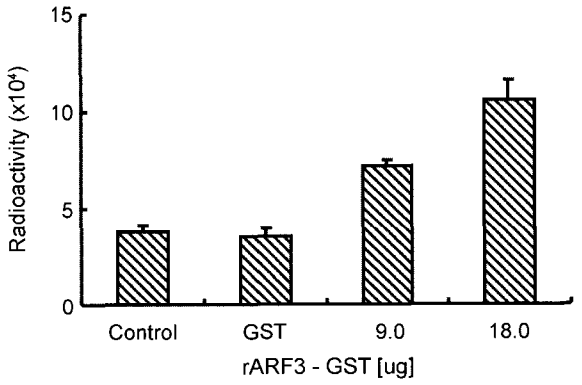

Fig. 5. GTP binding activity of rARF3 in guinea pig lung tissues.
The functional activity of recombinant ARF (rARF)-GST fusion protein was assessed by quantification of GTPγS binding, as described in Materials and Methods. Bar indicates [^{35}S] GTPγS binding.

18.0 μg rARF) (Fig. 5). The activity of rARF3 from guinea pig lung was similar to the native ARF activity (purified ARF), as shown in Fig. 2b.

DISCUSSION

A signal-activated phospholipase D (PLD) in several tissues and cell lines including mast cells [16, 25, 38] has been described. This enzyme activity is stimulated by two subfamilies of small GTP-binding proteins such as ADP-ribosylation factor (ARF) [8, 11] and Rho A family [5], and classical isoforms of protein kinase C (PKC) [36]. Interestingly, studies from numerous laboratories have linked these factors to secretary and exocytotic processes [6, 23, 41]. PLD and ARF are also known to play an important role in signal transduction in mast cells that are major effector cells in allergic reactions [42].

PLD and ARF have been characterized in many tissues. However, these proteins from guinea pig lung tissues have not yet been purified and characterized. Therefore, we purified these proteins to confirm whether PLD and ARF are present in guinea pig lung tissue, which is very similar to the human airway system, in order to characterize the ARF and PLD-mediated signaling pathway in allergic reactions.

Our experimental results showed that PLD localized in guinea pig lung tissue was very low and very sensitive to temperature. Therefore, it was partially purified by two columns chromatography (Fig. 1a), and the activity of PLD was high although the recovery rate was very low (Table 1). Two major active PLD peaks were found in the chromatography of Heparin Sepharose column, however, PLD activity was not found (data not shown) in either of the protein fractions that were eluted with 55% salt buffer via High-Q ion-exchange column, but it was found in one protein peak eluted with 15% salt buffer. The second active PLD fraction eluted from the Heparin Sepharose column showed two protein peaks eluted from High Q column, but PLD activity was not found. This might have been due to a difference in the amino acid sequence or to another PLD isotype that is unknown. Therefore, further study is needed to elucidate the biochemical property and physiological functions of these proteins. The molecular size of PLD in immunoblotting, estimated to be 64 kDa and 85 kDa, may be smaller than any other mammalian PLD isoform (90–120 kDa). Two bands of PLD in immunoblotting analysis may be PLD isotypes such as PLD1 and PLD2 (Fig. 3a). It has been reported that PLD was activated in the presence of ARF (mainly PLD1 and PLD2) and oleate (mainly PLD2) [19, 22, 40, 43]. In the case of PLD purified from guinea pig lung tissues, it was activated by ARF and oleate (Fig. 2a) in a dose-dependent manner. Therefore, it can be inferred that the function of membraneous PLD localized in guinea pig lung tissue is similar to that of PLDs (PLD1 and PLD2) localized in other mammalian tissues. This result also agrees with our previous report that the PLD enzyme was activated by specific antigen-antibody reaction in guinea pig lung mast cells [38].

ARFs in guinea pig lung cytosol showed high activity when purified by Uno-Q ion-exchange chromatography (Fig. 1b, Table 1). In the case of ARF purification, two bands of 29 kDa and 35 kDa were found in immunoblotting (Fig. 3b). Six isotypes of ARF(ARF 1-6), which have slightly different functions, have been reported [6, 11, 42]. In our results, two bands may be ARF isotypes. The partially purified ARF also activated the partially purified guinea pig lung PLD in a dose-dependent manner more than rARF (human neutrophil-derived rARF) did (Fig. 2b). This may be due to difference of organs and species. In contrast to PLD, the molecular sizes of guinea pig lung ARF were larger than those of other mammalian tissues [2, 4]. Nucleotide sequence identity between human ARF3 and guinea pig ARF3 showed 92% similarity, and amino acid sequence identity was 100%. ARF3 activity showed a native ARF activity-like pattern. The ARF3 has 91% identity with rat nucleotide sequence, and 92% identity with mouse nucleotide sequence. We partially isolated ARF1 from guinea pig lung tissue (data not shown). The partial sequence of ARF1 was found to have 89% identity with human ARF1 nucleotide sequence and 99% identity with the human ARF1 amino acid sequence. These results suggest that the PLD is localized in the membrane of guinea pig lung tissue, and the enzyme is activated by cytosolic small G-protein ARF and unsaturated fatty acid oleate.

Until now, it has not been fully characterized whether ARF purified from guinea pig lung tissue are involved in the regulation of PLD activity, and whether guinea pig lung ARF has amino acid sequence homology with human ARF. Therefore, we suggest that guinea pig lung PLD activity can be regulated by cytosolic ARF proteins similar to other species, although the size of these proteins differs from that found in other mammalian tissue. The results further suggest that the ARF proteins isolated from guinea pig lung tissue are ARF3 and ARF1, which have amino acid sequence homology with human ARF3.

Therefore, it is necessary to identify the full amino acid sequence of ARF and to characterize the biochemical properties of PLD isozymes in order to understand the regulatory role of PLD and ARF proteins as a factor in guinea pig lung allergic reactions. This will be a significant password for elucidating the mechanisms of allergic reaction in human lung.

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