Role of phospholipase A_2 enzymes in degradation of dipalmitoylphosphatidylcholine by granular pneumocytes

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Abstract The role of phospholipase A_2 (PLA_2) enzymes in the degradation of internalized dipalmitoylphosphatidylcholine (DPPC) by rat granular pneumocytes was evaluated with cells in 24 h primary culture on microporous membranes. In cell sonicates and rat lung homogenates, the transition state analogue MJ33 inhibited acidic (pH 4), Ca^{2+}-independent PLA_2 (aiPLA$_2$) while *p*-bromophenacylbromide (pBPB) inhibited alkaline (pH 8.5), Ca^{2+}-dependent PLA_2 and phospholipase C activities. With intact cells, degradation of [^{3}H]methylcholine-labeled DPPC during 2 h incubation was inhibited 48% by MJ33, 20% by pBPB, and 69% by the combination. The inhibitors (20 μM pBPB, 3 mol% MJ33) had no effect on cellular dye exclusion, adherence to membranes, or uptake of DPPC. Arachidonyl trifluoromethylketone, a cytoplasmic PLA_2 inhibitor, had no effect on cellular degradation of DPPC. Degradation was depressed approximately 20% by the addition of NH_4Cl or methylamine to the medium, suggesting a role for an acidic intracellular compartment in DPPC metabolism. Subcellular fractions prepared by differential centrifugation of rat lung homogenates showed highest specific activity of aiPLA$_2$ in the lamellar body and lysosomal fractions, lower activity in cytosol, and essentially no activity in mitochondria, microsomes, or plasma membranes. The results of this study indicate that aiPLA$_2$ has the major role in the degradation of internalized DPPC by granular pneumocytes and they are compatible with participation of lysosomes/lamellar bodies in DPPC metabolism.—**Fisher, A. B., and C. Dodia.** Role of phospholipase A_2 enzymes in degradation of dipalmitoylphosphatidylcholine by granular pneumocytes. *J. Lipid Res.* 1996. **37:** 1057–1064.

Supplementary key words MJ33 • *p*-bromophenacylbromide • phospholipase C • lamellar bodies • lysosomes • microsomes • subcellular fractions • liposomes • Ca^{2+}-independent PLA_2

Dipalmitoylphosphatidylcholine (DPPC), the major lipid component of lung surfactant, is primarily responsible for surfactant effects on alveolar surface tension. DPPC is synthesized and secreted by granular pneumocytes, the cuboidal epithelial cells of the alveolar septum (1). Granular pneumocytes also endocytose and degrade DPPC and are thus responsible for the total metabolism of this surfactant phospholipid (2). Degradation of approximately 60–70% of internalized DPPC during 2 h incubation of isolated granular pneumocytes (2–4) is compatible with the known rapid DPPC turnover in the lung (1).

Phospholipase A_2 (PLA_2) activity, the pathway for the major fraction of DPPC degradation in granular pneumocytes, represents a diverse family of enzymes (5). Types 1 and 2 secreted PLA_2 (sPLA$_2$) are small (approx. 14 kDa), Ca^{2+}-dependent enzymes with pH optimum at approx. 8.5 and sensitivity to *p*-bromophenacylbromide (pBPB) (5). Cytosolic PLA_2 (cPLA$_2$) is a larger protein (approx. 85 kDa) that requires μM Ca^{2+} for binding to substrate, shows preference for PC with arachidonate in the *sn*-2 position, and is inhibited by hydrated fluoroketones, such as arachidonyl trifluoromethylketone (AACOCF$_3$) (5). Both type 2 sPLA$_2$ (6) and cPLA$_2$ (7, 8) have been identified in lungs. Ca^{2+}-independent PLA_2 (iPLA$_2$) has recently been described and appears to represent a separate class of enzyme (5). We have isolated a Ca^{2+}-independent PLA_2 from rat lung (9) that shows maximal activity at acidic pH (pH 4), a property that appears to differentiate it from other iPLA$_2$ enzymes. We have given this enzyme the designation aiPLA$_2$. aiPLA$_2$ is insensitive to pBPB and to AACOCF$_3$, but is inhibited by the transition state analogue, 1-hexadecyl-3-trifluoroethylglycero-*sn*-2-phosphomethanol (MJ33) (9). The purpose of the present study was to compare the effects of these PLA_2 inhibitors on the

Abbreviations: DPPC, dipalmitoylphosphatidylcholine or 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine; PLA$_2$, phospholipase A$_2$; sPLA$_2$, secreted phospholipase A$_2$; cPLA$_2$, cytoplasmic phospholipase A$_2$; iPLA$_2$, Ca^{2+}-independent phospholipase A$_2$; aiPLA$_2$, acidic Ca^{2+}-independent phospholipase A$_2$; pBPB, *para*-bromophenacylbromide; MJ33, 1-hexadecyl-3-trifluoroethylglycero-*sn*-2-phosphomethanol; AACOCF$_3$, arachidonyl trifluoromethylketone; MEM, minimal essential medium; FCS, fetal calf serum.

metabolism of DPPC by isolated granular pneumocytes in order to evaluate the relative roles of PLA_2 classes in lung surfactant lipid turnover.

MATERIALS AND METHODS

Lung cell isolation

Granular pneumocytes were isolated from rat lungs by previously described methods (10). Lungs were cleared of blood by perfusion through the pulmonary artery, lavaged with modified phosphate-buffered saline, and incubated with elastase solution instilled through the trachea. Lungs were minced and agitated to obtain a crude lung cell preparation that was panned on IgG-precoated bacteriologic plastic plates followed by overnight culture on Transwell tissue culture-treated microporous membranes (24.5 mm diameter, 0.4 μm pore size, CoStar) in minimal essential medium (MEM) plus 10% fetal calf serum (FCS) at pH 7.4 (Gibco/BRL). Purity of the granular pneumocyte preparation exceeded 90% by specific staining with phosphine 3R with macrophages as the primary contaminant. To prepare cell sonicates, cells were removed from Transwell membranes by treatment with 0.02% EDTA for 10 min and then 0.05% trypsin in 0.02% EDTA for an additional 10 min. Cells were pelletted, resuspended in saline, and disrupted by two 15-sec bursts with a probe sonicator at 60% of maximum power (Sonic Dismembrator, Artek Systems Corp.). Alveolar macrophages were prepared by centrifugation (300 *g*) of fluid obtained from lavage of rat lungs with saline (5 × 7 ml).

Lung homogenate and subcellular fractions

Methods for subcellular fractionation of lung have been described previously (11, 12) and are presented here briefly. Lungs from anesthetized rats (pentobarbital, 50 mg/kg i.p.) were cleared of blood by perfusion through the pulmonary artery. For PLA_2 assay, lungs were homogenized in saline (tissue:medium, 1:10 by vol) by sequential use of a Polytron with P-10 probe and Potter-Elvehjem vessel with a motor-driven pestle. For subcellular fractionation, lungs were homogenized in 0.32 M sucrose and used to isolate enriched fractions of mitochondria (10,000 *g* pellet) and plasma membrane, microsomes, and cytosol by sucrose-gradient differential centrifugation (11). A parallel sucrose gradient was used to isolate a lamellar body-enriched fraction by upward flotation (11). A lysosome-enriched fraction was isolated using Percoll gradient centrifugation (12). The enrichment factors in marker enzyme activities compared with the lung homogenate were approximately 14 for plasma membranes (5′-nucleotidase), 3 for mitochondria (rotenone-insensitive succinic dehydrogenase), 3 for microsomes (NADPH-cyto C reductase), 7 for lysosomes (aryl sulfatase), and 61 for lamellar bodies (phospholipid to protein ratio) (11, 12).

Preparation of liposomes

Lipids from stock solutions in chloroform were combined in the ratio of 0.5 mol DPPC, 0.25 mol egg PC, 0.1 mol phosphatidylglycerol, 0.15 mol cholesterol (all obtained from Avanti, Birmingham, AL) with tracer radiolabeled DPPC (New England Nuclear). The radiolabeled DPPC was either *sn*-2 [9,10-^{3}H]palmitate or [^{3}H]*methyl*choline. The specific activity of DPPC in the lipid mixture was 4400 DPM/nmol using either label. The lipid mixture was evaporated to dryness under N_2, resuspended in buffer, and subjected to freeze-thaw procedure three times by alternating liquid N_2 and warm water. Liposomes of approximately 0.1–0.2 μm in diameter were prepared by ten sequential rapid extrusions through polycarbonate membranes (0.1 μm pore size) using 17–20 atm pressure from an N_2 gas cylinder. The liposomes were either used immediately or stored overnight at 4°C. For studies with MJ33, the inhibitor was added to the lipid mixture prior to the freeze-thaw procedure; concentration of MJ33 was expressed as mol % of total lipid.

Uptake and degradation of DPPC by granular pneumocytes

Cells on Transwell membranes were incubated with radiolabeled liposomes ([^{3}H]choline label) at 80 μM DPPC (equivalent to 120 μM total PC) for 2 h at 37°C as previously described (4, 10). Incubations were carried out in the presence of 0.1 mM 8-BrcAMP, which has been shown previously to stimulate both liposome uptake and metabolism by granular pneumocytes (4) but does not directly affect $aiPLA_2$ activity (13). The reaction was started by addition of liposomal substrate to both the upper and lower compartments of the Transwell. MJ33, when used, was added to the lipid mixture as a component of the liposomal substrate. All other inhibitors were added to the cells and pre-incubated for 1 h prior to addition of liposomes.

At the end of 2 h incubation, cells were washed 3 times with MEM to remove free substrate and inhibitors. Cells were removed from membranes with trypsin/EDTA as described above. This latter treatment also serves to remove liposomes bound to the cell surface (14). Cells were pelletted by centrifugation at 1000 *g*, washed, and resuspended in saline. Aliquots were assayed for protein, total DPM by scintillation counting, and DPM in organic and aqueous fractions after extraction by the Bligh and Dyer method (15). The organic fraction was separated into lysoPC and PC components by TLC on silica gel G with the solvent system $CHCl_3$–CH_3OH–NH_4OH–H_2O

65:35:2.5:2.5 (by vol) (16, 17). Recovery of DPM in the lysoPC plus PC spots exceeded 95% of the counts applied to the plate. An aliquot of the organic fraction was osmicated and used to isolate disaturated PC by chromatography on a neutral alumina column (18). Disaturated PC will be referred to herein as DPPC, although some small fraction may have been contributed by saturated fatty acids other than palmitate. Unsaturated PC was calculated as total PC minus DPPC.

Uptake of DPPC was calculated from total cell-associated DPM at the end of incubation and degradation as total DPM minus DPM in DPPC. Recovery of DPM in the aqueous-soluble, lysoPC, and unsaturated PC fractions also was calculated. Results were calculated in terms of specific activity of DPPC in the original liposomes and expressed per mg cell protein.

Phospholipase assays

PLA_2 activity was assayed using the pH 4, Ca^{2+}-free and pH 8.5, plus Ca^{2+} assays as previously described (4, 16). For convenience, these are referred to as acidic and alkaline assay systems. The assays are linear with time and with protein (4). The acidic assay buffer was 40 mM Na acetate, 5 mM EDTA, pH 4.0. The alkaline assay buffer was 50 mM Tris-HCl, 1 mM EGTA, 10 mM $CaCl_2$, pH 8.5. The substrate was 1 mM DPPC, radiolabeled with palmitate in the *sn*-2 position, in unilamellar liposomes (total PC 1.5 mM). Assay was carried out in 1 ml reaction medium containing 300 μg lung homogenate protein or 200 μg cell sonicate protein. MJ33 was studied as a component of the liposomes, as described above. For studies with other inhibitors, homogenate was pre-incubated at pH 7.4 for 30 min at 37°C and then pH was adjusted with concentrated Na acetate or NaOH as appropriate. Reaction was started by the addition of liposomal substrate, continued for 60 min at 37°C, and terminated with 5 ml hexane–ether 1:1 (v/v). The organic layer was removed and the aqueous fraction was re-extracted with hexane–ether. The organic fractions were combined, evaporated to dryness, and separated into radiolabeled free fatty acid and diacylglyceride fractions by thin-layer chromatography using hexane–ether–acetic acid solvent system (16, 19). Authentic palmitic acid and diacylglyceride (Avanti) were co-chromatographed. In some experiments, recovery in lysoPC and phosphatidic acid was also measured. Appropriate spots were identified with I_2 vapor, scraped from the plates, and analyzed by scintillation counting using internal standards for quench correction. Total phospholipase activity was calculated from the disappearance of label in DPPC. PLA_2 activity was calculated from recovery of radiolabel in free fatty acids. Phospholipase C (PLC) activity was calculated from recovery of radiolabel in diacylglycerides. Activity was expressed in terms of DPPC hydrolyzed, calculated from the specific radioactivity of DPPC in liposomal substrate, and lung homogenate or cell sonicate protein. Protein was measured by the Coomassie blue binding assay with bovine gamma-globulin as standard (Bio-Rad Labs, Richmond, CA).

Statistical analyses

Most experiments were carried out in duplicate and the results were averaged. Results are expressed as mean ± SE (or mean ± range for n = 2) and were evaluated for statistical significance by analysis of variance (ANOVA) for multiple comparisons with Bonferroni correction using Sigma Stat (Jandel Scientific, San Rafael, CA). The level of statistical significance was taken as $P < 0.05$.

RESULTS

Phospholipase activity of granular pneumocytes

Total phospholipase activity at pH 4 (minus Ca^{2+}) of sonicated granular pneumocytes, measured by the disappearance of ^{3}H-dpm in *sn*-2 palmitate-labeled DPPC, was 45.2 ± 0.6 nmol in 1 h/mg protein (mean ± range, n = 2). Essentially 100% of radiolabel lost from DPPC was recovered in metabolic products. Recovery was 1.3 ± 0.1% in lysoPC, 69.5 ± 0.6% in free fatty acid, 27.8 ± 0.8% in diacylglycerol, and 1.3 ± 0.1% in phosphatidic acid. These results indicate activity of both PLA_2 and PLC but no significant activity of phospholipase A_1 (PLA_1) or phospholipase D (PLD) at acidic pH. With the pH 8.5 assay, total phospholipase activity was 53.8 ± 0.9 nmol in 1 h/mg protein (n = 2) and recovery of labeled metabolites was 9.5 ± 1.1% in lysoPC, 52.9 ± 0.6% in free fatty acid, 20.7 ± 1.1% in diacylglycerol, and 16.9 ± 0.5% in phosphatidic acid. These results indicate significant PLA_1 and PLD activity in addition to PLA_2 and PLC at the alkaline pH.

MJ33 and pBPB were used as inhibitors of PLA_2. As pBPB can alter cell viability, we evaluated the cellular

TABLE 1. Effect of pBPB concentration on granular pneumocyte phospholipase activity, erythrocin B exclusion, and cell protein recovery

pBPB Conc.	PLase Activity	Dye Exclusion	Recovered Cell Protein
μM	*nmol in 1 h/mg protein*	*% of cells*	*μg/well*
0	41.4	97	202
2	5.7	96	196
20	5.3	97	209
100	5.5	58	116

Cells were incubated at 37°C for 2 h with indicated concentration of pBPB. Aliquots of cells were analyzed for dye exclusion and sonicated for measurement of cell protein and total phospholipase (PLase) activity at pH 8.5.

TABLE 2. Phospholipase activities of sonicated granular pneumocytes

	PLA_2 Activity		PLC Activity	
	pH 4	pH 8.5	pH 4	pH 8.5
	nmol in 1 h/mg protein		*nmol in 1 h/mg protein*	
Control	24.6 ± 0.4	28.2 ± 0.6	12.3 ± 0.3	11.8 ± 0.3
+ MJ33	3.2 ± 0.6[a]	26.6 ± 0.8	12.4 ± 0.7	11.1 ± 0.1
+ pBPB	20.3 ± 0.3	0.9 ± 0.2[a]	12.4 ± 0.3	3.6 ± 0.1[a]
+ MJ33 + pBPB	2.9 ± 0.2[a]	1.0 ± 0.2[a]	-	-

Incubation was for 1 h at 37°C with 200 mg cell protein and 1 mM [^{3}H]DPPC in liposomes (total PC 1.5 mM). The radiolabel was [9,10-^{3}H]palmitate in position *sn*-2 of DPPC. MJ33 was 3 mol% and pBPB 20 μM. For study of pBPB, the cell sonicate was pre-incubated with inhibitor for 30 min at pH 7.4. Values are mean ± SE for n = 3.

[a] $P < 0.05$ vs. corresponding control.

effects of varying concentrations of this inhibitor. Intact granular pneumocytes were incubated for 2 h with pBPB, washed, and trypsinized from the membranes as described above, sonicated, and then analyzed for total phospholipase activity at pH 8.5 (plus Ca^{2+}). Phospholipase activity was inhibited by 86% with 2 μM pBPB and was not inhibited further with higher concentrations (**Table 1**). The effect of pBPB on phospholipase activity was similar when cells were sonicated prior to addition of the inhibitor (data not shown). Viability (dye exclusion) of cultured granular pneumocytes was evaluated at the end of 2 h incubation with pBPB (Table 1). For this study, granular pneumocytes were cultured on 35-mm plastic dishes to permit microscopic visualization. For control conditions (no BPB), nearly all of the cells excluded the dye, erythrosin B, and the percentage was unchanged with 2 or 20 μM pBPB. Cell protein recovery from the Transwell culture wells also was unaffected by these concentrations of inhibitor, indicating that pBPB did not lead to detachment of cells from the membranes. By contrast, 100 μM pBPB led to significant decreases in dye exclusion and cell protein recovery indicating cellular toxicity at this concentration (Table 1). Further studies of DPPC metabolism by intact cells were carried out using 20 μM pBPB. MJ33 was studied at 3 mol% and $AACOCF_3$ at 100 μM, neither of which altered dye exclusion by cells or recovery of protein from culture wells (data not shown).

Phospholipase A_2 activity

PLA_2 activity of sonicated granular pneumocytes was measured using the standard acidic (Ca^{2+}-free) and alkaline (plus Ca^{2+}) assays. Acidic PLA_2 was inhibited 87% by 3 mol% MJ33 while alkaline PLA_2 was unaffected (**Table 2**). MJ33 produced maximal inhibition of acidic PLA_2 in both intact and sonicated cells at 1 mol% (**Fig. 1**) as previously described for the isolated perfused lung (16). pBPB (20 μM) inhibited alkaline PLA_2 by 97% but had no effect on acidic PLA_2 (Table 2). Thus, these two inhibitors could be used to differentiate between acidic, Ca^{2+}-independent and alkaline, Ca^{2+}-dependent PLA_2 activities. pBPB also significantly inhibited PLC activity measured in the alkaline assay while this activity was not inhibited by MJ33 under either assay condition (Table 2).

Degradation of DPPC

Granular pneumocytes were evaluated for degradation of DPPC presented to the cells in multicomponent unilamellar liposomes. During a 2-h incubation under control conditions (no inhibitors but in the presence of

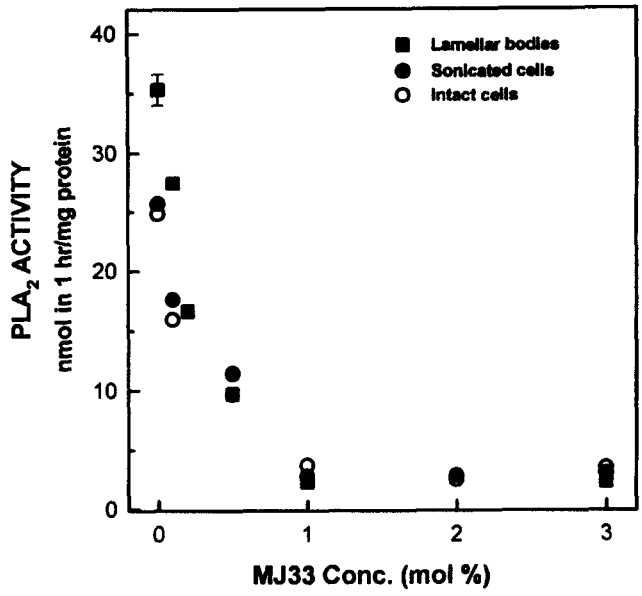

Fig. 1. Effect of MJ33 concentration on acidic, Ca^{2+}-independent PLA_2 activity. Rat granular pneumocytes were cultured for 24 h on Transwell membranes. PLA_2 activity in the presence of varying MJ33 concentration was measured in sonicated granular pneumocytes and in lamellar bodies isolated from rat lung. In additional experiments, cells on membranes (intact cells) were incubated for 2 h with MJ33 in non-labeled liposomes and then washed, trypsinized, sonicated, and assayed for PLA_2 activity. The substrate was *sn*-2 [9,10-^{3}H]palmitate-labeled DPPC. The varying concentration of MJ33 is expressed as mol% of total lipid.

TABLE 3. Uptake and degradation of [^{3}H]DPPC by granular pneumocytes

	Uptake	Degradation		Recovery of Metabolites
	μg DPPC in 2 h/mg protein	μg DPPC in 2 h/mg protein	% of Control	μg DPPC in 2 h/mg protein
Control	4.75 ± 0.05	3.10 ± 0.04	-	3.01 ± 0.05
+ MJ33	4.55 ± 0.03	1.63 ± 0.03[a]	52.5	1.66 ± 0.07[a]
+ pBPB	4.77 ± 0.05	2.47 ± 0.01[a,b]	79.7	2.43 ± 0.07[a,b]
+ MJ33 + BPB	4.78 ± 0.10	0.97 ± 0.06[a,b]	31.2	0.91 ± 0.06[a,b]
+ $AACOCF_3$	4.86 ± 0.01	3.18 ± 0.02	102	3.17 ± 0.02

Cells were incubated at 37°C for 2 h with 0.8 mM [^{3}H]DPPC-containing liposomes (0.12 mM total PC) and 0.1 mM 8-BrcAMP plus or minus inhibitors (3 mol% MJ33, 20 μM pBPB, 100 μM $AACOCF_3$). For study of pBPB, cells were pre-incubated with the inhibitor for 60 min. DPPC radiolabel was in choline. Degradation represents loss of label in DPPC. Recovery of metabolites represents ^{3}H recovery in aqueous-soluble fraction plus lysoPC plus unsaturated PC. Results are mean ± SE for n = 3–4.

[a] $P < 0.05$ vs. corresponding control.

[b] $P < 0.05$ for pBPB ± MJ33 vs. MJ33.

0.1 mM 8-BrcAMP), granular pneumocytes degraded approximately two-thirds of the internalized DPPC (**Table 3**). There was no effect of either MJ33 or pBPB on uptake of DPPC although degradation was inhibited significantly. MJ33 inhibited DPPC degradation by 47% while pBPB inhibited by 20%; the effect of these inhibitors was additive (Table 3). Incubation with 50 μM pBPB (data not shown) did not increase the extent of inhibition compared with the 20 μM concentration, indicating a maximal pBPB effect. The presence of $AACOCF_3$ had no effect on DPPC uptake or degradation by the cells (Table 3).

The distribution of DPPC metabolites after a 2-h incubation was measured. As shown previously (2, 3), recovery of radiolabeled metabolites of choline-labeled DPPC was greatest in the aqueous-soluble fraction (**Table 4**). Radiolabel in this fraction has been identified previously as glycerophosphorylcholine, choline phosphate, CDP-choline, and free choline (2). An additional significant fraction of radiolabel was recovered in unsaturated PC and a small component in lysoPC (Table 4). In the original liposomes, 100% of radiolabel was in DPPC (not shown). The total recovery of radiolabel in metabolic products (Table 4) showed good correlation to the measured loss of label in DPPC (Table 3). This analysis of degradation does not take into account the possible metabolic re-utilization of degradation products for resynthesis of radiolabeled DPPC.

Results for distribution of metabolites (Table 4) are expressed as percentage of DPPC uptake; as uptake was unchanged with the inhibitors, the percentages reflect DPPC degradation. In the presence of MJ33, recovery of aqueous soluble radiolabeled metabolites significantly decreased while recovery in unsaturated PC and lysoPC was unchanged compared with control. The effects of pBPB on the recovery of aqueous soluble metabolites was less marked (Table 4).

Based on the effect of MJ33, the major fraction of DPPC degradation appeared to be mediated by aiPLA$_2$, an enzyme with acidic pH optimum (9). Therefore, we evaluated the effect of lysosomotrophic agents on DPPC metabolism. Incubation of cells with NH_4Cl or methylamine had no effect on DPPC uptake. However, there was a small (approximately 20%) but significant decrease in DPPC degradation (**Table 5**). This result is compatible with decreased phospholipase activity due to organellar alkalinization. This concentration of inhibitors had no effect on the % of cells that excluded erythrocin B (data not shown). As the presence of residual NH_4Cl or methylamine could have affected recovery of metabolic products, we added these agonists at 1 and 10 mM to the cell sonicate after 1 h incubation under control (no agonists)

TABLE 4. Distribution of ^{3}H-labeled metabolites after 2 h incubation of granular pneumocytes with [^{3}H]DPPC

	Recovery of ^{3}H dpm, % of [^{3}H]DPPC Uptake			
	Total Metabolites	LysoPC	Aqueous	Unsaturated PC
Control	65.2 ± 0.3	2.5 ± 0.6	42.6 ± 1.6	20.1 ± 0.8
+ MJ33	35.8 ± 1.1[a]	2.0 ± 0.4	10.2 ± 0.4[a]	23.5 ± 1.7
+ pBPB	51.7 ± 0.6[a,b]	1.6 ± 0.4	30.5 ± 1.8[a,b]	19.6 ± 0.7
+ MJ33 + pBPB	20.3 ± 1.0[a,b]	0.9 ± 0.3[a]	3.5 ± 0.4[a,b]	15.3 ± 0.8[b]
+ $AACOCF_3$	65.4 ± 0.7	2.3 ± 0.3	43.0 ± 0.5	20.1 ± 0.4

Results were obtained with the experiments shown in Table 3.

[a] $P < 0.05$ vs. corresponding control.

[b] $P < 0.05$ for pBPB ± MJ33 vs. MJ33.

TABLE 5. Effect of lysosomotrophic agents on uptake and degradation of DPPC by granular pneumocytes

	Uptake	Degradation
	μg DPPC in 2 h/mg protein	μg DPPC in 2 h/mg protein
Control	4.74 ± 0.06	3.21 ± 0.01
NH_4Cl, 10 mM	4.68 ± 0.05	2.59 ± 0.03[a]
Methylamine, 10 mM	4.63 ± 0.10	2.48 ± 0.02[a]

Cells were pre-incubated for 1 h with lysosomotrophic agents and then incubated with [^{3}H]DPPC-labeled liposomes under conditions as described in Table 3. Results are mean ± SE for n = 3.
[a] $P < 0.05$ vs. control.

conditions. There was no significant effect of either NH_4Cl or methylamine on the recovery of DPPC metabolites or estimation of DPPC degradation (data not shown).

Phospholipase activity of alveolar macrophages

Since alveolar macrophages contaminated the granular pneumocyte preparation, their phospholipase activity also was measured in sonicated cells. The presence of an acidic, Ca^{2+}-independent PLA_2 activity in alveolar macrophages has been demonstrated previously (20). In the presence of pBPB, alveolar macrophage alkaline phospholipase activity was decreased from 12.9 ± 0.8 (control) to 2.4 ± 0.02 nmol in 1 h/mg protein (n = 2). Acidic phospholipase activity of macrophages was 12.0 ± 0.2 nmol in 1 h/mg protein and decreased to 6.3 ± 0.1 in the presence of MJ33 (n = 4). Macrophage $aiPLA_2$ activity (calculated as MJ33 sensitive activity) was 5.7 ± 0.3 nmol in 1 h/mg protein, or less than 30% of granular pneumocyte activity (compare with Table 2). As contamination of the cell preparation with macrophages was less than 10%, their presence would not significantly influence the measured $aiPLA_2$ activity of granular pneumocytes.

Subcellular localization of $aiPLA_2$

Subcellular fractionation studies for $aiPLA_2$ activity were carried out with rat lung homogenate. PLA_2 activity with the acidic assay in the homogenized lung was 17.6 ± 0.2 nmol in 1 h/mg protein and decreased to 2.5 ± 0.3 with MJ33 (n = 3). Acidic, Ca^{2+}-independent PLA_2 activity was detected in each of the subcellular fractions that were studied (**Table 6**). However, the activities in mitochondria, microsomes and plasma membranes were unaffected by MJ33 while the activities in lamellar body, lysosomal, and cytosolic fractions were markedly inhibited (Table 6). The specific activity of $aiPLA_2$ (defined as MJ33-sensitive activity using the acidic assay) was greatest in lamellar bodies. The MJ33 concentration dependence for inhibition of acidic PLA_2 activity of isolated lamellar bodies and of sonicated granular pneumocytes was similar (Fig. 1).

DISCUSSION

This study has evaluated the degradation of DPPC, the major phospholipid component of lung surfactant, by granular pneumocytes in primary culture and specifically the role of PLA_2. These cells accumulated DPPC-labeled liposomes from the medium and extensively degraded the internalized lipid as shown by loss of label in DPPC and recovery of radiolabel in metabolic products. Degradation of DPPC requires activity of one or more phospholipases.

Previous studies have indicated that the full range of phospholipases (PLA_1, PLA_2, PLC, PLD) is present in lung tissue (6–9, 21–23) and conceivably could participate in DPPC degradation. The present study has shown the presence of these activities in isolated granular pneumocytes. With choline-labeled DPPC used for incubation of intact cells, the primary product of both PLA_1 and PLA_2 activities would be labeled lysoPC while PLC activity would generate labeled choline phosphate and PLD would generate labeled choline. In the present study, the bulk of recovered radiolabel was in aqueous-soluble products, either due to activity of PLC/PLD or to the subsequent metabolism of lysoPC by lysophospholipases and related enzymes. Radiolabel recovery in the aqueous-soluble fraction was markedly (76%) decreased by the presence of MJ33, a specific PLA_2 inhibitor, providing evidence that the aqueous-soluble metabolites were in large part derived from lysoPC. Recovery in unsaturated PC, representing reutilization of degradation products, could occur through de novo PC synthesis from radiolabeled free choline (or the phosphate) or through reacylation of lysoPC with an unsaturated fatty acid.

PLA_2 activity represents a diverse family of enzymes (5). $sPLA_2$, $cPLA_2$, and $iPLA_2$ all have been identified in lungs (8, 9, 23) and $iPLA_2$ (present study) and $cPLA_2$ (7) are present in granular pneumocytes. The relative roles of various PLA_2 activities in degradation of internalized DPPC by granular pneumocytes was evaluated through use of inhibitors. In cell sonicates, MJ33 only inhibited

TABLE 6. Acidic PLA_2 activity in lung subcellular fractions

	PLA_2 Activity, nmol in 1 h/mg protein		
	Control	+ MJ33	MJ33 Sensitive
Lamellar bodies	31.2 ± 0.5	4.4 ± 0.4	26.8 ± 1.0
Lysosomes	15.9 ± 0.4	3.4 ± 0.1	12.5 ± 0.2
Mitochondria	8.8 ± 0.4	8.3 ± 0.1	0.5 ± 0.3
Microsomes	19.5 ± 0.4	18.2 ± 0.1	1.3 ± 0.4
Plasma membranes	7.3 ± 0.2	6.8 ± 0.3	0.5 ± 0.2
Cytosol	10.9 ± 0.1	2.0 ± 0.1	8.9 ± 0.2

Results are mean ± range for n = 2. Assay was at pH 4 and 37°C for 1 h with 50 μg protein for lamellar bodies and lysosomes and 200 μg for other fractions. MJ33 (3 mol%) sensitive activity was calculated by subtraction.

PLA_2 activity in the acidic assay while pBPB inhibited PLA_2/PLC activities in the alkaline assay. Both MJ33 and pBPB decreased degradation of DPPC by intact cells. The greater decrease with MJ33 is compatible with previous results (4, 16) suggesting that $aiPLA_2$ plays the major role accounting for approximately 50% of DPPC degradation by granular pneumocytes. DPPC degradation was inhibited by approximately 20% in the presence of pBPB. As pBPB inhibited PLC as well as $sPLA_2$ activities in the sonicated cells, its effect on DPPC degradation in the intact cells could be through inhibition of either enzyme. The observed additive effect of MJ33 and pBPB supports a role for at least two different enzyme activities in DPPC degradation. Approximately 30% of DPPC degradation was uninhibited by the presence of these PLA_2 inhibitors suggesting a role for other phospholipases in this metabolic activity. $AACOCF_3$ had no effect on DPPC metabolism in intact cells so that significant involvement of $cPLA_2$ in degradation of internalized DPPC by granular pneumocytes is unlikely. The lack of $AACOCF_3$ effect cannot be explained by membrane impermeability as this inhibitor also had no effect on PLA_2 activity of sonicated cells (data not shown) and it has been shown to be membrane permeable with other cultured cell systems (24). These results do not exclude a role for $cPLA_2$ in granular pneumocyte cellular signalling events, especially related to arachidonyl-containing phospholipids as opposed to the dipalmitoyl substrate used in the present study.

The subcellular site for DPPC degradation by $aiPLA_2$ can be inferred based on its requirements for activity. Using subcellular fractions derived from homogenized lungs, $aiPLA_2$ activity was identified in lamellar bodies, lysosomes, and cytosol. As isolated $aiPLA_2$ is inactive at pH > 6 (9), a physiologic role for cytosolic activity would not be expected. Further, it is possible that the "cytosolic" activity represents enzyme released from organelles (lamellar bodies/lysosomes) as previously described for the lysosomal PLA_2 activity of alveolar macrophages (20). Therefore, degradation of DPPC by $aiPLA_2$ likely occurs in the acidic lamellar body/lysosomal compartments (25). A role for an acidic intracellular compartment is supported by the observed inhibition of DPPC degradation in the presence of the lysosomotrophic agents NH_4Cl and methylamine. Acidic, Ca^{2+}-independent PLA_2 activity also was demonstrated in mitochondria, microsomes, and plasma membrane preparations but was MJ33 insensitive and may represent a different enzyme, albeit a relatively minor component of total cellular PLA_2.

In summary, we have shown that MJ33 and pBPB are useful probes to differentiate between $aiPLA_2$ and other phospholipases in the metabolism of DPPC by isolated granular pneumocytes. Inhibition of $aiPLA_2$ with MJ33 results in marked decrease in the degradation of internalized liposomal DPPC while the effects of pBPB were significantly less. Subcellular fractionation studies and the effects of MJ33 suggest that the major site of DPPC degradation in these cells is the lysosomes and/or the lamellar bodies. These results provide evidence for a significant role for $aiPLA_2$ and an acidic subcellular compartment in the metabolism of internalized lung surfactant lipid.

We thank Dr. Mahendra Jain for advice concerning the use of PLA_2 inhibitors, Dr. Michael Gelb for the supply of AACOCF3, and Elaine Primerano for typing the manuscript. This work was presented in part at the annual meeting of the American Society for Cell Biology, San Francisco, CA, December 1994. Support was provided by HL19737 from the National Institutes of Health.

Manuscript received 3 November 1995 and in revised form 2 February 1996.

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