Role of phospholipase A₂ enzymes in degradation of dipalmitoylphosphatidylcholine by granular pneumocytes

Aron B. Fisher and Chandra Dodia
Institute for Environmental Medicine, University of Pennsylvania Medical Center, Philadelphia, PA 19104

Abstract The role of phospholipase A₂ (PLA₂) 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 tran-

choline (DPPC) by rat granular pneumocytes was evaluated with cells in the degradation of internalized combination. The inhibitors 

sition state analogue MJ33 inhibited acidic (pH 4), Ca²⁺-independent PLA₂ (aiPLA₂) while p-bromophenacylbromide (pBPB) inhibited alkaline (pH 8.5), Ca²⁺-dependent PLA₂ and phospholipase C activities. With intact cells, degradation of [³H]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 cyto-

toplasmic PLA₂ inhibitor, had no effect on cellular degradation of DPPC. Degradation was depressed approximately 20% by the addition of NH₄Cl or methylamine to the medium, sug-

gesting a role for an acidic intracellular compartment in DPPC metabolism. Subcellular fractions prepared by differ-

ential centrifugation of rat lung homogenates showed highest specific activity of aiPLA₂ 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 aiPLA₂ 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 A₂ 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 • Ca²⁺-independent PLA₂

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 A₂ (PLA₂) 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 PLA₂ (sPLA₂) are small (approx. 14 kDa), Ca²⁺-dependent enzymes with pH optimum at approx. 8.5 and sensitivity to p-bromophenacylbromide (pBPB) (5). Cytosolic PLA₂ (cPLA₂) is a larger protein (approx. 85 kDa) that requires μM Ca²⁺ for binding to substrate, shows preference for PC with arachidonic in the sn-2 position, and is inhibited by hydrated fluoro-
tones, such as arachidonyl trifluoromethylketone (AA-

COCFs) (5). Both type 2 sPLA₂ (6) and cPLA₂ (7, 8) have been identified in lungs. Ca²⁺-independent PLA₂ (iPLA₂) has recently been described and appears to represent a separate class of enzyme (5). We have iso-

lated a Ca²⁺-independent PLA₂ from rat lung (9) that shows maximal activity at acidic pH (pH 4), a property that appears to differentiate it from other iPLA₂ en-
zymes. We have given this enzyme the designation aiPLA₂. aiPLA₂ is insensitive to pBPB and to AACOCFs, but is inhibited by the transition state analogue, 1-

hexadecyl-3-trifluoroethyglycero-sn-2-phosphomethanol (MJ33) (9). The purpose of the present study was to compare the effects of these PLA₂ inhibitors on the
metabolism of DPPC by isolated granular pneumocytes in order to evaluate the relative roles of PLA₂ 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 pelleted, 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 PLA₂ 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 [9,10-3H]palmitate or [3H]methylcholine. The specific activity of DPPC in the lipid mixture was 4400 DPM/nmol using either label. The lipid mixture was evaporated to dryness under N₂, resuspended in buffer, and subjected to freeze-thaw procedure three times by alternating liquid N₂ 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 N₂ 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 ([3H]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 aPLA₂ 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 pelleted 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 CHCl₃–CH₃OH–NH₄OH–H₂O.
Phospholipase assays

PLA₂ activity was assayed using the pH 4, Ca²⁺-free and pH 8.5, plus Ca²⁺ 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 CaCl₂, 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 I₂ 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. PLA₂ 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 Ca²⁺) of sonicated granular pneumocytes, measured by the disappearance of ³H-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 PLA₂ and PLC but no significant activity of phospholipase A₁ (PLA₁) 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 PLA₁ and PLD activity in addition to PLA₂ and PLC at the alkaline pH.

MJ33 and pBPB were used as inhibitors of PLA₂. As pBPB can alter cell viability, we evaluated the cellular

<table>
<thead>
<tr>
<th>TABLE 1. Effect of pBPB concentration on granular pneumocyte phospholipase activity, erythbrobin B exclusion, and cell protein recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBPB Conc.</td>
</tr>
<tr>
<td>µM</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>100</td>
</tr>
</tbody>
</table>

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 (PLₐ) activity at pH 8.5.
TABLE 2  Phospholipase activities of sonicated granular pneumocytes

<table>
<thead>
<tr>
<th>Activity</th>
<th>pH 4</th>
<th>pH 8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA$_2$</td>
<td>nmol in 1 h/mg protein</td>
<td>nmol in 1 h/mg protein</td>
</tr>
<tr>
<td>Control</td>
<td>24.6 ± 0.4</td>
<td>28.2 ± 0.6</td>
</tr>
<tr>
<td>+ MJ33</td>
<td>3.2 ± 0.5</td>
<td>26.6 ± 0.8</td>
</tr>
<tr>
<td>+ pBPB</td>
<td>20.3 ± 0.3</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>+ MJ33 + pBPB</td>
<td>2.9 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
</table>

Incubation was for 1 h at 37°C with 200 mg cell protein and 1 mM [3H]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.

**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
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 AACOCF3 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 the 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 aiPLA2, an enzyme with acidic pH optimum (9). Therefore, we evaluated the effect of lysosomotropic agents on DPPC metabolism. Incubation of cells with NH4Cl or methy- lamine 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 NH4Cl or methy- lamine 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 3. Uptake and degradation of [3H]DPPC by granular pneumocytes

<table>
<thead>
<tr>
<th></th>
<th>Uptake</th>
<th>Degradation</th>
<th>Recovery of Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg DPPC in 2 h/mg protein</td>
<td>µg DPPC in 2 h/mg protein</td>
<td>% of Control</td>
</tr>
<tr>
<td>Control</td>
<td>4.75 ± 0.05</td>
<td>3.10 ± 0.04</td>
<td>-</td>
</tr>
<tr>
<td>+ MJ33</td>
<td>4.55 ± 0.03</td>
<td>1.63 ± 0.03</td>
<td>52.5</td>
</tr>
<tr>
<td>+ pBPB</td>
<td>4.77 ± 0.05</td>
<td>2.47 ± 0.01</td>
<td>79.7</td>
</tr>
<tr>
<td>+ MJ33 + pBPB</td>
<td>4.78 ± 0.10</td>
<td>0.97 ± 0.06</td>
<td>81.2</td>
</tr>
<tr>
<td>+ AACOCF3</td>
<td>4.86 ± 0.01</td>
<td>3.18 ± 0.02</td>
<td>102</td>
</tr>
</tbody>
</table>

Cells were incubated at 37°C for 2 h with 0.8 mM [3H]DPPC-containing liposomes (0.12 µmol total PC) and 0.1 mM 8-BrcAMP plus or minus inhibitors (5 mM MJ33, 20 µM pBPB, 100 µM AACOCF3). 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 % recovery in aqueous-soluble fraction plus lysoPC plus unsaturated PC. Results are mean ± SE for n = 3–4.

*P < 0.05 vs. corresponding control.

*P < 0.05 for pBPB ± MJ33 vs. MJ33.

### Table 4. Distribution of [3H]-labeled metabolites after 2 h incubation of granular pneumocytes with [3H]DPPC

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Control</th>
<th>MJ33</th>
<th>pBPB</th>
<th>MJ33 + pBPB</th>
<th>AACOCF3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LysoPC</td>
<td>2.5 ± 0.6</td>
<td>2.0 ± 0.4</td>
<td>1.6 ± 0.4</td>
<td>0.9 ± 0.3</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>Aqueous</td>
<td>42.6 ± 1.6</td>
<td>10.2 ± 0.4</td>
<td>30.5 ± 1.8</td>
<td>3.5 ± 0.4</td>
<td>43.0 ± 0.5</td>
</tr>
<tr>
<td>Unsaturated PC</td>
<td>20.1 ± 0.8</td>
<td>23.5 ± 1.7</td>
<td>19.6 ± 0.7</td>
<td>15.3 ± 0.8</td>
<td>20.1 ± 0.4</td>
</tr>
</tbody>
</table>

Results were obtained with the experiments shown in Table 3.

*P < 0.05 vs. corresponding control.

*P < 0.05 for pBPB ± MJ33 vs. MJ33.
Phospholipase activity was decreased from 12.9 fAcidic phospholipase activity of macrophages was 12.0 lar pneumocyte preparation, their phospholipase activ-

ity also was measured in sonicated cells. The presence of an acidic, Ca2+-independent PLA2 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 6.5 ± 0.1 in the presence of MJ33 (n = 4). Macrophage aiPLA2 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 aiPLA2 activity of granular pneumocytes.

Subcellular localization of aiPLA2

Subcellular fractionation studies for aiPLA2 activity were carried out with rat lung homogenate. PLAz 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, Ca2+-independent PLAz 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 aiPLA2 (defined as MJ33-sensitive activity using the acidic assay) was greatest in lamellar bodies. The MJ33 concentration dependence for inhibition of acidic PLAz 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 PLAz. 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 (PLAz, PLAl, 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 PLAz and PLA2 activities would be labeled lysophosphatidylcholine while PLC activity would generate labeled choline phosphatidylcholine 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 lysophosphatidylcholine by lysophospholipases and related enzymes. Radiolabel recovery in the aqueous-soluble fraction was markedly (76%) decreased by the presence of MJ33, a specific PLAz inhibitor, providing evidence that the aqueous-soluble metabolites were in large part derived from lysophosphatidylcholine. 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 lysophosphatidylcholine with an unsaturated fatty acid.

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

| TABLE 6. Acidic PLAz activity in lung subcellular fractions |
|-----------------|-----------------|-----------------|
| PLAz 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.
PLA₂ activity in the acidic assay while pBPB inhibited PLA₂/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 aiPLA₂ 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 sPLA₂ 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 enzymes in DPPC degradation. Approximately 30% of DPPC degradation was uninhibited by the presence of either enzyme. The observed additive effect of MJ33 and pBPB suggests a role for other phospholipases in this metabolic activity. AACOCF₃ had no effect on DPPC metabolism in intact cells so that significant involvement of cPLA₂ in degradation of internalized DPPC by granular pneumocytes is unlikely. The lack of AACOCF₃ effect cannot be explained by membrane impermeability as this inhibitor also had no effect on PLA₂ 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 cPLA₂ in granular pneumocyte metabolism, especially related to arachidonate-containing phospholipids as opposed to the dipalmitoyl substrate used in the present study.

The subcellular site for DPPC degradation by aiPLA₂ can be inferred based on its requirements for activity. Using subcellular fractions derived from homogenized lungs, aiPLA₂ activity was identified in lamellar bodies, lysosomes, and cytosol. As isolated aiPLA₂ 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 PLA₂ activity of alveolar macrophages (20). Therefore, degradation of DPPC by aiPLA₂ likely occurs in the acidic lamellar body/lysosomal compartiments (25). A role for an acidic intracellular compartment is supported by the observed inhibition of DPPC degradation in the presence of the lysosomotropic agents NH₄Cl and methylamine. Acidic, Ca²⁺-independent PLA₂ 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 PLA₂.

In summary, we have shown that MJ33 and pBPB are useful probes to differentiate between aiPLA₂ and other phospholipases in the metabolism of DPPC by isolated granular pneumocytes. Inhibition of aiPLA₂ 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 aiPLA₂ and an acidic subcellular compartment in the metabolism of internalized lung surfactant lipid.

We thank Dr. Mahendra Jain for advice concerning the use of PLA₂ 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.

REFERENCES


