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Calcium-Independent Phospholipase A2 Is Required for Lysozyme Secretion in U937 Promonocytes

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As a part of their surveillance functions in the immune system, monocytes/macrophages secrete large amounts of the bactericidal enzyme lysozyme to the extracellular medium. We report here that lysozyme secretion in activated U937 promonocytes depends on a functional calcium-independent phospholipase A2 (iPLA2). Inhibition of the enzyme by bromoenol lactone or by treatment with a specific antisense oligonucleotide results in a diminished capacity of the cells to secrete lysozyme to the extracellular medium. Calcium-independent PLA2 is largely responsible for the maintenance of the steady state of lysophosphatidylcholine (lysoPC) levels within the cells, as manifested by the marked decrease in the levels of this metabolite in cells deficient in iPLA2 activity. Reconstitution experiments reveal that lysoPC efficiently restores lysozyme secretion in iPLA2-deficient cells, whereas other lysophospholipids, including lysophosphatidic acid, lysophosphatidylserine, and lysophosphatidylethanolamine, are without effect. Arachidonic acid mobilization in activated U937 cells is under control of cytosolic phospholipase A2 (cPLA2). Selective inhibition of cPLA2 results in a complete abrogation of the arachidonate mobilization response, but has no effect on lysozyme secretion. These results identify iPLA2-mediated lysoPC production as a necessary component of the molecular machinery leading to lysozyme secretion in U937 cells and rule out a role for cPLA2 in the response. Collectively, the results demonstrate distinct roles in inflammatory cell signaling for these two intracellular phospholipases. The Journal of Immunology, 2003, 170: 5276–5280.

Lysozyme degrades bacterial cell walls of Gram-positive bacteria and the chitinous components of fungal cell walls. The enzyme occurs in many body fluids, such as tears, saliva, and mucus, and is produced and secreted by phagocytic cells and a variety of cells of epithelial origin (1). Stimuli that induce lysozyme secretion from phagocytic cells also induce the phospholipase A2 (PLA2)-mediated mobilization of free arachidonic acid (AA). Whether these two responses are causally related has been the subject of recent research (2–4).

PLA2 enzymes are frequently classified into three main classes on the basis of whether the enzyme is secreted (sPLA2), cytosolic Ca2+-dependent (cPLA2), or cytosolic Ca2+-independent (iPLA2) (5, 6). The sPLA2s are low molecular mass, secreted enzymes that require millimolar concentrations of calcium for their catalytic activity and do not show fatty acid selectivity (7). The cPLA2 is an 85-kDa protein that requires nanomolar to micromolar concentrations of calcium, is specific for AA-containing phospholipids, and appears to play a crucial role in agonist-induced AA mobilization (8). The iPLA2 has been shown to play in important role in regulating basal phospholipid deacylation/reacylation reactions in phagocytic cells (9).

U937 promonocytic cells are derived from a human histiocytic lymphoma and can be activated by a variety of agonists, such as phorbol myristate acetate (PMA) (10, 11). U937 cells express cPLA2 (12) and iPLA2 (13), but not sPLA2 (14). The objective of the current study was to determine the involvement of cPLA2 and iPLA2 in lysozyme release and AA mobilization in activated U937 cells. We report that U937 cells deficient in iPLA2 activity show a decreased capacity to secrete lysozyme. On the contrary, cPLA2 inhibition has no effect on lysozyme secretion, but abrogates AA release. Together, the results suggest that iPLA2 and cPLA2 play distinct roles in regulating the proinflammatory responses of activated U937 promonocytes.

Materials and Methods

Reagents

[5,6,8,9,11,12,14,15-3H]AA (sp. act., 100 Ci/mmol) and [methyl-3H]choline chloride (sp. act., 79 Ci/mmol) were obtained from NEN (Boston, MA). [2-14C]Ethanolamine (sp. act., 57 mCi/mmol) was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). Bromoelactone, [6,6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (BEL), and methyl arachidonyl fluorophosphonate (MAFP) were purchased from Cayman Chemicals (Ann Arbor, MI). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO). The specific cPLA2 inhibitor pyrrophenone was provided by Dr. K. Seno (Shionogi, Osaka, Japan) (15).

Cell culture

U937 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) FCS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). For experiments, the cells were incubated at 37°C in a humidified atmosphere of CO2/1% CO2 at a cell density of 0.5–1 x 10^6 cells/ml in 12-well plastic culture dishes (Costar, Cambridge, MA).

Treatment of the cells with antisense oligonucleotides

The antisense oligonucleotides used in these studies were derived from prior publications reporting their effects (16–18). The iPLA2 antisense sequence corresponded to nt 59–78 in the murine group VI iPLA2 sequence, which is conserved in human group VI iPLA2 (19, 20). The antisense sequence corresponded to nt 59–78 in the murine group VI iPLA2 sequence, which is conserved in human group VI iPLA2 (19, 20).
sequence was 5′-CTC TTT CAC CCG GAA TGG GT-3′. As a control, the iPLA2 sense sequence was 5′-ACC CAT TCC GGG TGA AGG AG-3′.

Phosphorothiate-modified oligonucleotides were used to limit degradation. The antisense and sense oligonucleotides were mixed with Lipofectamine (Invitrogen, Carlsbad, CA), and complexes were allowed to form at room temperature for 15–30 min. The complexes were then added to the cells, and the incubations were allowed to proceed under standard cell culture conditions. The final concentrations of oligonucleotide and Lipofectamine were 1 μM and 10 μg/ml, respectively. Oligonucleotide treatment and culture conditions were not toxic for the cells as assessed by the trypan blue dye exclusion assay and by quantitating adherent cell protein.

Lysozyme release assay

The cells were stimulated with PMA, Con A, or platelet-activating factor (PAF) for the indicated times. After centrifugation, the supernatants were removed, and the cell pellets were overlaid with 1 ml of PBS and homogenized. Lysozyme in the supernatants and the cell pellets was measured spectrophotometrically as follows. Briefly, 1 ml of sample was mixed with 1 ml of a *Micrococcus luteus* suspension (0.3 mg/ml in 0.1 M sodium phosphate buffer, pH 7.0). The decrease in absorbance at 450 nm was measured at room temperature. A calibration curve was constructed using chicken egg white lysozyme as a standard. Lysozyme release is expressed according to the formula: 

\[
\text{S/S} + P \times 100,
\]

where S is the amount of lysozyme measured in the supernatant, and P is the amount of lysozyme measured in the cell pellets.

Measurement of AA release

The cells were labeled with 0.5 μCi/ml [3H]AA for 18 h. After this period, the cells were washed and placed in serum-free medium for 1 h before the addition of 100 nM PMA in the presence of 0.5 mg/ml BSA. The supernatants were removed, cleared of cells by centrifugation, and assayed for radioactivity by liquid scintillation counting.

Lyosphospholipid determination

Cells labeled with 0.5 μCi/ml [3H]choline or [14C]ethanolamine for 2 days were used. After the incubations, lipids were extracted with ice-cold n-butanol and separated by TLC with chloroform/methanol/acetic acid/water (50/40/60/6) as a solvent system. Spots corresponding to lysoosphatidylcholine (lysoPC) or lysophosphatidylethanolamine (lysoPE) were scraped into scintillation vials, and the amount of radioactivity was estimated by liquid scintillation counting (16).

Data presentation

Assays were conducted in duplicate or triplicate. Each set of experiments was repeated at least three times with similar results. Unless otherwise indicated, the data presented are from representative experiments.

Results

Lysozyme release in U937 cells

Lysozyme is one of the proteases involved in nonspecific immune defense against bacterial infection. Fig. 1 shows that the U937 cells released large quantities of lysozyme to the incubation medium when activated with the phorbol ester PMA. More than 60% of total enzyme was found in the supernatant after 2-h incubation with PMA. Fig. 1 also shows that lysozyme secretion was strongly blunted by the PLA2 inhibitors BEL and MAFP, suggesting the possible involvement of iPLA2 and/or cPLA2 in this response.

To test the above suggestion more rigorously, the effect of an iPLA2 antisense oligonucleotide on lysozyme secretion was evaluated. The antisense oligonucleotide used is the human counterpart of the murine one that we and others have successfully employed previously (16–18). In these experiments this antisense produced a 70–80% decrease in both immunoreactive iPLA2 protein and cellular iPLA2 activity (data not shown, but see Ref. 21). Under these conditions, lysozyme release in PMA-treated cells was strongly inhibited (Fig. 2), thus providing strong evidence for the involvement of iPLA2 in this process.

The cPLA2 inhibition was achieved by the use of pyrrophenone, a highly selective inhibitor of cPLA2 vs iPLA2 in cells (15, 22). Pyrrophenone exerted no significant effect on the release of lysozyme (Fig. 1).

Studies were conducted next to evaluate the effect of iPLA2 inhibition on lysozyme release in response to the receptor-mediated agonists Con A and PAF. The significant release of lysozyme induced by both agonists was strongly abrogated by BEL (Fig. 3). These data indicate that iPLA2 inhibition also leads to modulation of receptor-mediated lysozyme release in U937 promonocytes.

**FIGURE 1.** Lysozyme secretion from activated U937 cells. The cells were treated with [■] or without [□] 100 nM PMA for 2 h in the absence or presence of 25 μM BEL, 25 μM MAFP, or 10 μM pyrrophenone (Pyr), as indicated. Lysozyme secretion was quantitated as described in Materials and Methods.

**FIGURE 2.** The iPLA2 antisense oligonucleotide inhibits lysozyme secretion in U937 cells. The cells were either untreated (control) or treated with sense (SiPLA2) or antisense (ASiPLA2) oligonucleotides as indicated. Lysozyme secretion was measured in the supernatants of cells treated [■] or untreated [□] with 100 nM PMA for 2 h.

**FIGURE 3.** The iPLA2 inhibition blocks lysozyme secretion by receptor agonists. The cells were treated with 100 μg/ml Con A, 1 μM PAF, or neither (control) as indicated, in the absence [□] or the presence [■] of 25 μM BEL. Lysozyme secretion was determined as described in Materials and Methods.
AA mobilization in activated U937 cells is not involved in lysozyme release

[3H]AA-labeled U937 cells were stimulated with the phorbol ester PMA (100 nM) for different periods of time, and the release of radiolabel in the supernatant was measured. After a time lag of ~15 min, PMA-treated cells showed a modest, but significant, release of radiolabel (Fig. 4). This sustained release was completely blocked by MAFP, but was unaffected by BEL (Fig. 4), suggesting that, in agreement with previous data (13, 21), cPLA2, not iPLA2, mediates AA release in activated U937 promonocytic cells.

The cPLA2-mediated AA release was sensitive to the unspecific kinase inhibitor quercetin (23). Fifty percent inhibition was observed at a quercetin concentration of 5 μM, while higher concentrations of the inhibitor were required to inhibit lysozyme secretion (Fig. 5). The lack of correspondence between the concentration-response effects of quercetin on AA release and lysozyme secretion suggests that both responses are unrelated.

LysoPC levels are decreased in iPLA2-deficient cells

The iPLA2 plays a major role in a number of cells in the regulation of basal phospholipid deacylation reactions by providing the bulk of lysoPC acceptors used in these reactions (9, 24). Fig. 6A shows that U937 cells made deficient in iPLA2 by antisense treatment exhibited considerably lesser amounts of lysoPC than control cells, and this was readily observable in control unstimulated cells as well as in PMA-stimulated U937 cells. Changes in cellular lysoPC due to activation with PMA were too small to be detected (not shown). No significant effect of the iPLA2 antisense on cellular levels of lysoPE in [14C]ethanolamine-labeled cells was detected.

Importantly, addition of lysoPC (25 μM) restored lysozyme release in the iPLA2-deficient cells stimulated with 100 nM PMA. Other lysophospholipids tested, i.e., lysoPE, lysophosphatidylserine, and lysophosphatic acid were ineffective. LysoPAF was as effective as lysoPC in restoring lysozyme release (Fig. 6B). Together, these results suggest that it is the choline headgroup of the lysophospholipid that is necessary for biological activity under these settings. The dose response of the effect of lysoPC on the restoration of lysozyme release

FIGURE 4. Effects of BEL and MAFP on PMA-induced AA release. The cells were treated with 25 μM BEL (●), 25 μM MAFP (▲), or neither (○) for 30 min before the addition of 100 nM PMA. ○, Control incubations, i.e., those that received neither PMA nor inhibitors. The inhibitors alone did not significantly affect the control release.

FIGURE 5. Effect of quercetin on AA release and lysozyme secretion in activated U937 cells. The cells were incubated with the indicated concentrations of quercetin for 30 min before the addition of 100 nM PMA. AA release (●) and lysozyme secretion (○) were measured as described in Materials and Methods. To allow for direct comparison, the data are presented as a percentage of the responses obtained in the absence of the inhibitor.

FIGURE 6. LysoPC effects on lysozyme secretion in U937 cells. A, Effect of iPLA2 antisense oligonucleotide on lysoPC levels. The cells were labeled with [3H]choline at the time they were treated with oligonucleotides. The cellular amount of lysoPC in control, sense-treated (SiPLA2), or antisense-treated (ASiPLA2) cells was determined as described in Materials and Methods. B, LysoPC restores lysozyme secretion in BEL-treated cells. Cells deficient in iPLA2 activity by treatment with 25 μM BEL were incubated without (○) or with (●) 100 nM PMA in the presence of lysoPC (LPC), lysoPE (LPE), lysophosphatidylserine (LPS), lysophosphatidic acid (LPA), or lysoPAF (LPAF) for 2 h, as indicated. Afterward lysozyme secretion was measured as described in Materials and Methods. C, Dose response of the effect of lysoPC on the restoration of lysozyme secretion by iPLA2-deficient cells stimulated with 100 nM PMA.
release in activated cells is shown in Fig. 6C. Significant effects of lysoPC were already observed at concentrations between 5–10 μM. LysoPC-mediated phospholipid concentrations >25 μM induced significant lysozyme release on their own and thus were not suitable for these reconstitution experiments.

Addition of free fatty acids such as AA, palmitic acid, or linoleic acid (up to 10 μM) failed to restore lysozyme release in iPLA2-deficient U937 cells (not shown). Collectively, the results suggest that lysoPC, and not other putative PLA2-derived metabolites such as a free fatty acid or lysoPE, is required for U937 cells to release lysozyme to the incubation medium.

Studies of the regulation of iPLA2 activity
Homogenates of U937 cells, either untreated or treated with PMA, were prepared, and assays were conducted to assess iPLA2 activity using a vesicle substrate assay. Under these conditions we failed to detect any change in the iPLA2 specific activity of homogenates from PMA-treated cells vs untreated cells. As an alternative approach, iPLA2 was measured using the mammalian membrane assay system described by Diez and colleagues (25). We have previously used this system to detect iPLA2 activity changes in homogenates from H2O2-treated U937 cells (21). In this system, purified [3H]AA-labeled mammalian membranes are used as a substrate. Using this assay, again no differences in the iPLA2 activity of untreated cells vs that of PMA-treated cells could be demonstrated.

Discussion
The current study addresses the possible involvement of two intracellular PLA2s in lysozyme secretion in activated U937 cells. In particular, the data identify iPLA2 as an important player in the secretion process and rule out a significant role for cPLA2. These conclusions are based on two different approaches that yield cells deficient in either iPLA2 activity or cPLA2 activity, namely the use of chemical inhibitors and antisense oligonucleotides. Thus, experimental approaches leading to the blockade of cellular iPLA2 result in abrogation of the cell’s capacity to secrete lysozyme. On the contrary, strategies that lead to the blockade of cPLA2 activity do not significantly affect lysozyme release.

The iPLA2 appears to play an important role in regulating phospholipid fatty acid recycling in a variety of cell types by providing the lysoPC that is a natural amphiphile; it incorporates into membranes and affects membrane fluidity and permeability (32–34). For instance, lysoPC alters the surface charge of the plasma membrane and affects membrane fluidity and permeability (32–34). LysoPC is a natural amphiphile; it incorporates into membranes and affects membrane fluidity and permeability (32–34).

An interesting aspect of these results, showing the importance of lysoPC in lysozyme secretion, is the possibility of functional redundancy with exogenous secreted PLA2s that might act on the lysozyme-secreting cells in a paracrine fashion. Certain PLA2 forms that are secreted by immunoinflammatory cells are able to attack the outer membrane phosphatidylcholine very well. These receptors for lysoPC that mediate these actions of the phospholipid have recently been described (36). Signaling through lysoPC receptors involves rapid activation of the mitogen-activated protein kinase cascade as well as increased intracellular Ca2+ levels (36). However, exogenous lysoPC neither activates the mitogen-activated protein kinase cascade nor increases the intracellular Ca2+ levels in U937 cells in the presence of the mitogen-activated protein kinase inhibitor quercetin. In turn, iPLA2 plays no role in stimulus-induced AA mobilization in these cells. It is interesting to note that cPLA2 activation transiently elevates cellular lysoPC levels in activated cells (37). Since cPLA2 activation transiently elevates cellular lysoPC levels in activated cells, these results show the importance of lysoPC in lysozyme secretion. In agreement with this observation, no changes in the specific activity of iPLA2 were detected after activation of the cells with PMA.

An interesting aspect of these results, showing the importance of lysoPC in lysozyme secretion, is the possibility of functional redundancy with exogenous secreted PLA2s that might act on the lysozyme-secreting cells in a paracrine fashion. Certain PLA2 forms that are secreted by immunoinflammatory cells are able to attack the outer membrane phosphatidylcholine very well. These include group X PLA2 (38, 39) and also group V PLA2 (40). The temporal accumulation of lysoPC at discrete sites on the plasma membrane induced by these enzymes might facilitate the exocytotic process.
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