Calcium-independent phospholipase A2 mediates proliferation of human promonocytic U937 cells

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The phospholipase A2 (PLA2) superfamily is a heterogeneous group of enzymes with distinct roles in cell function [1–5]. The common feature of these enzymes is that they all selectively hydrolyze the fatty acid at the sn-2 position of glycerophospholipids. However, it is becoming increasingly clear that PLA2s differ with respect to substrate specificity, co-factor requirements for activity, and cellular localization [1–5]. Mammalian cells usually contain several PLA2s, and thus the challenge in recent years has been to ascribe specific cellular functions to particular PLA2 forms. PLA2s are systematically classified into several groups, many of which include various subgroups [5]. However, based on their biochemical commonalities, PLA2s are usually grouped into four major families, namely Ca2+-dependent enzymes, Ca2+-dependent cytosolic enzymes (cPLA2), Ca2+-independent cytosolic enzymes (iPLA2), and platelet-activating factor acetyl hydrolases [1,5].

We have investigated the possible involvement of two intracellular phospholipases A2, namely group VIA calcium-independent phospholipase A2 (iPLA2-VIA) and group IVA cytosolic phospholipase A2 (cPLA2-a), in the regulation of human promonocytic U937 cell proliferation. Inhibition of iPLA2-VIA activity by either pharmacological inhibitors such as bromoenol lactone or methyl arachidonyl fluorophosphonate or using specific antisense technology strongly blunted U937 cell proliferation. In contrast, inhibition of cPLA2-a had no significant effect on U937 proliferation. Evaluation of iPLA2-VIA activity in cell cycle-synchronized cells revealed highest activity at G2/M and late S phases, and lowest at G1. Phosphatidylcholine levels showed the opposite trend, peaking at G1 and lowest at G2/M and late S phase. Reduction of U937 cell proliferation by inhibition of iPLA2-VIA activity was associated with arrest in G2/M and S phases. The iPLA2-VIA effects were found to be independent of the generation of free arachidonic acid or one of its oxygenated metabolites, and may work through regulation of the cellular level of phosphatidylcholine, a structural lipid that is required for cell growth/membrane expansion.

The iPLA2 family consists of two members in mammalian cells, designated iPLA2-VIA and iPLA2-VIB, of which the former is the best characterized [3,6,7]. Since its purification [8] and cloning [9,10] in the mid-1990s, iPLA2-VIA has attracted considerable interest due to the multiple roles and functions that this enzyme may have in cells. Several splice variants of iPLA2-VIA co-exist in cells, and thus it is conceivable that multiple regulation mechanisms exist for this enzyme, which may depend on cell type. Thus, iPLA2-VIA may be a multi-faceted enzyme with multiple functions of various kinds (i.e. homeostatic, catabolic and signaling) in different cells and tissues [3,7].

Several lines of evidence have suggested a key role for iPLA2-VIA in control of the levels of phosphatidylcholine (PC) in cells by regulating basal deacylation/reacylation reactions. This is manifested by the significant reduction in the steady-state level of lysoPC that is observed shortly after acute inhibition of

**Abbreviations**

AA, arachidonic acid; BEL, bromoenol lactone; cPLA2-a, group IVA cytosolic phospholipase A2; iPLA2-VIA, group VIA calcium-independent phospholipase A2; MAFP, methyl arachidonyl fluoromethyl phosphonate; PC, phosphatidylcholine; PL2a, phospholipase A2.
iPLA₂-VIA by treatment of cells with bromoenol lactone (BEL) [11,12]. In INS-1 insulinoma cells, acute inhibition of iPLA₂-VIA reduces the relatively high content of lysoPC of these cells by about 25% [12], and the decrease is about 50–60% in macrophage-like cell lines [11,13,14], suggesting that the dependence of PC metabolism on iPLA₂-VIA may vary from cell to cell. In some cell types, particularly (but not uniquely) phagocytes [11,13–19], reduction of the steady-state level of lysoPC slows the initial rate of incorporation of exogenous arachidonic acid (AA) into cellular phospholipids. In other studies, it has been shown that iPLA₂-VIA may be coordinately regulated with CTP:phosphocholine cytidylyltransferase to maintain PC levels [20–23]. Given that PC is the major cellular glycerophospholipid present in mammalian cell membranes and thus plays a key structural role, we hypothesized that iPLA₂-VIA may play an important role in processes such as cell proliferation for which membrane phospholipid biogenesis is required. Thus we studied the possible involvement of iPLA₂-VIA in the normal proliferative response of human promonocytic U937 cells, and compared it to that of another major intracellular PLA₂, the AA-selective cPLA₂. Utilizing various strategies, we demonstrate here that iPLA₂-VIA, but not cPLA₂, plays a key role in U937 cell proliferation by a mechanism that does not involve AA or one of its oxygenated metabolites.

Results

iPLA₂ inhibition slows U937 cell proliferation

Using RT-PCR, we have previously found that human promonocytic U937 cells express both cPLA₂ and iPLA₂-VIA, but, strikingly, do not express Group V, Group X or any of the group II secreted PLA₂s [24]. Enzymatic activities corresponding to both cPLA₂ and iPLA₂-VIA are readily detected in the U937 cells by utilizing specific enzyme assays and inhibitors [18,25]. We began the current study by investigating whether the activities of these two intracellular phospholipases are required for normal U937 cell growth (i.e. that induced by the serum present in the culture medium, in the absence of any other mitogenic stimulus). First, the effect of various selective PLA₂ inhibitors was examined. Figure 1 shows that the selective cPLA₂ inhibitor pyrrophenone [26] completely blocked the Ca²⁺-dependent PLA₂ activity of U937 cell homogenates at concentrations as low as 0.5–1 µM. However, at these concentrations, pyrrophenone failed to exert any effect on the proliferation of U937 cells, as measured by a colorimetric staining assay (Fig. 1).

In contrast to pyrrophenone, the iPLA₂ inhibitor BEL strongly blocked the growth of the U937 cells (Fig. 2). In these experiments, a BEL concentration of 5 µM was utilized to avoid the pro-apoptotic effect of this drug when used at higher concentrations [27–29]. We confirmed that, at 5 µM, BEL significantly blunted cellular iPLA₂ activity, as measured by an in vitro assay (Fig. 2). Collectively, the data in Figs 1 and 2 are consistent with the involvement of iPLA₂, but not cPLA₂, in U937 cell proliferation. Owing to the lack of specificity of BEL in cell-based assays [28], additional pharmacological evidence for the involvement of iPLA₂ in U937 cell growth was obtained using methyl...
arachidonyl fluoromethyl phosphonate (MAFP), a dual iPLA₂/cPLA₂ inhibitor that is structurally unrelated to BEL and pyrrophenone [30,31]. Concentrations of MAFP that completely inhibited cellular Ca²⁺-independent PLA₂ activity also led to strong inhibition of U937 cell growth (Fig. 3). Given that the pyrrophenone experiments had established that cPLA₂ is not critical for U937 cell growth, the inhibitory effect of MAFP seen in Fig. 3 can be attributed to inhibition of iPLA₂.

To confirm iPLA₂ involvement in U937 cell growth in a more definitive manner, the effect of an antisense oligonucleotide directed against iPLA₂-VIA was evaluated. In these experiments, the antisense construct produced a 70–75% decrease in immunoreactive iPLA₂-VIA and markedly inhibited (30–40%) U937 cell proliferation (Fig. 4).

**Inhibition of iPLA₂ does not induce cell death**

Trypan blue assays after the various treatments leading to iPLA₂ inhibition indicated no loss of viability, suggesting that necrotic cell death did not occur. To examine the possibility of apoptotic cell death, we...
utilized the annexin V-binding assay, which measures externalization of phosphatidylserine, a marker of apoptosis. Incubation of the U937 cells with 10 μM MAFP or 5 μM BEL for 24 h, conditions that result in inhibition of iPLA₂ activity and cell growth as shown above (Figs 2 and 3), had no effect on the number of annexin V-positive cells, which always remained below 12% of the total cell population. Antisense inhibition of iPLA₂-VIA also did not increase the number of annexin V-positive cells. As a control for these experiments, we also studied the effect of a higher BEL concentration, i.e. 25 μM, which is known to induce apoptotic cell death in U937 cells in an iPLA₂-independent manner [27]. Confirming our previous data, 25 μM BEL increased the extent of apoptotic cell death to well above 75% after a 24 h incubation period. Together, these data indicate that the slowed growth of cells deficient in iPLA₂ activity by either pharmacological or antisense methods arises as a result of slowed cell division and not increased apoptosis.

**iPLA₂ activity during the cell cycle**

To obtain more information on the role of iPLA₂ on U937 cell growth, we used flow cytometry to examine the cell-cycle dependence of iPLA₂ activity in the U937 cells. The cells were synchronized with nocodazole [23,32] and then allowed to progress through the cell cycle under normal culture conditions. Immediately after release from the mitotic block with nocodazole, more than 75% of the cells were in the G₂/M phase (Fig. 5). The cells were in G₁ from 2–8 h after release from nocodazole, and in S phase thereafter up to 10 h. After 10 h, the cells became largely asynchronous again. Thus, this method allows study of the cell cycle of U937 cells in G₂/M throughout the G₁ and S phases [23,32].

iPLA₂ activity measurements during the cell cycle revealed significant differences depending on the phase (Fig. 5). Highest activity was found during G₂/M, decreasing as the cells entered G₁ and then increasing again as the cells approached and entered S phase. The same pattern of variation of iPLA₂ activity was detected whether the assay was conducted with mixed micelles, vesicles or natural membranes as substrates (not shown), thus confirming the biological relevance...
of the findings. Quantification of the levels of PC, the major membrane phospholipid in mammalian cells, during the cell cycle showed a pattern that was clearly opposite to that found for iPLA₂ activity (Fig. 5). PC levels rose abruptly in early G₁ and then slowly declined as the cells progressed into late G₁ and S (Fig. 5). That PC levels and iPLA₂ activity show opposite kinetics is fully consistent with the possibility that iPLA₂ behaves as a major regulator of PC catabolism, which is responsible for glycerophospholipid accumulation during the cell cycle [23,33]. Thus, decreased iPLA₂ activity during the G₁ phase would result in an increase in PC content due to reduced catabolism.

**Induction of cell cycle arrest by iPLA₂ inhibition**

Having established that iPLA₂ activity is cell-cycle-regulated, and that its levels inversely correlate with those of the major membrane phospholipid PC, we set out to investigate whether the slowed growth due to iPLA₂ inhibition was a consequence of cell-cycle arrest. The cells were synchronized with nocodazole and then treated with BEL to inhibit iPLA₂ activity. Pyrrophenone was also used as a control. Figure 6 shows that treatment of the cells with BEL induced a significant accumulation of cells in G₂/M and S, and a concomitant decrease of cells in G₁, with respect to untreated cells. In contrast, pyrrophenone induced no significant changes in the phase distribution (Fig. 6). Thus these data suggest that inhibition of iPLA₂, but not cPLA₂, causes cell arrest in S and G₂/M phases.

**Arachidonic acid and/or its metabolites are not involved in U937 cell growth**

In addition to its role in PC homeostasis, iPLA₂, as a sn-2 lipase, may also participate in generating free fatty acids such as AA, which could subsequently be metabolized to eicosanoids. The importance of AA and the eicosanoids as growth factors for various cell types has previously been demonstrated [34]. We tested first the effects of various cyclooxygenase and lipoxygenase inhibitors on the growth of U937 cells under normal culture conditions. The inhibitors employed were acetylsalicylic acid (up to 25 μM), indomethacin (up to 25 μM), NS-398 (up to 10 μM), ebselen (up to 10 μM), baicalein (up to 10 μM), MK-886 (up to 10 μM) and nordihydroguaiaretic acid (up to 10 μM). Control experiments had indicated that, at the concentrations employed, these inhibitors effectively blocked AA oxygenation by the cyclooxygenase and lipoxygenase pathways. None of the inhibitors exerted any significant effect on U937 cell growth (data not shown). We next studied whether adding 10 μM AA to the cell cultures attenuates the antiproliferative effect of inhibiting iPLA₂ by BEL or antisense technology. However, the results indicated that AA failed to restore the growth of cells deficient in iPLA₂ activity. Moreover, when the cells were synchronized with nocodazole, subsequent addition of exogenous AA exerted no detectable effect on the observed phase distribution (see Fig. 6), whether the cells had been treated with BEL or not (not shown). Collectively, these results suggest that AA or a metabolite does not mediate the effect of iPLA₂ on U937 cell proliferation.

**Discussion**

In this study, we demonstrate that iPLA₂-VIA is required for the proliferation of human promonocytic U937 cells under normal culture conditions (i.e. in the absence of any mitogenic stimulus other than serum), and that inhibition of iPLA₂-VIA by either pharmacological means or antisense technology slows growth by inducing arrest at the S and G₂/M phases. Cell accumulation at these phases of the cell cycle could not be reversed by supplying the medium with exogenous AA, indicating that the role of iPLA₂-VIA is not mediated via AA-derived mitogenic signaling. We also show that U937 cell iPLA₂-VIA activity is regulated in a cell-cycle-dependent manner, with maximal activity at G₂/M, steadily declining during G₁, and increasing again in late S phase. Strikingly, the levels of PC, the major membrane phospholipid in mammalian membranes, exhibit the opposite kinetics, with the highest levels at G₁. This inverse relationship between the kinetics of iPLA₂-VIA activity and PC accumulation agrees with previous studies in Jurkat cells [32] and CHO-K1 cells [23]. It is well established that changes in PC content during the cell cycle correlate better with the kinetics of its catabolism rather than synthesis [23,33,35], and the involvement of iPLA₂-VIA in the homeostatic regulation of membrane phospholipid turnover is one of the first roles attributed to this enzyme in cells [6,7]. Thus our results are in line with a scenario whereby iPLA₂-VIA plays a central role in cell growth and division by regulating glycerophospholipid metabolism during the cell cycle [23,32,36]. Thus, down-regulation of iPLA₂-VIA activity in G₁ and early S phase would allow accumulation of phospholipid in preparation for future cell division. Once cells enter S phase, the level of iPLA₂-VIA begins to increase, which would slow down phospholipid accumulation. It is interesting to note, however, that iPLA₂-VIA might not always be the major regulator of phospholipid catab-
Our data suggest that, 2 h after cell cycle entry, iPLA₂ is drastically reduced but PC levels appear to barely change (Fig. 5), raising the possibility that, at this time, involvement of fatty acid-reacylating enzyme activities or inter-phospholipid/diacylglycerol transacylation might be significant in regulating PC levels.

Whether, in addition to regulating glycerophospholipid metabolism during the cell cycle, iPLA₂-VIA may also act by activating receptor-mediated mitogenic signalling, e.g. by directly mediating the generation of lipid mediators with growth factor-like properties, is also a possibility that deserves consideration. Although we and others [23,32,37] have found no evidence for the

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**Fig. 6.** Effect of BEL and pyrrophenone on the U937 cell cycle. The cells were synchronized with nocodazole as described in Experimental procedures. After releasing the cells from the nocodazole block, they were untreated (open symbols) or treated with 5 μM BEL (closed symbols, left column) or 1 μM pyrrophenone (closed symbols, right column), and the percentage of cells at various phases of the cell cycle was studied by flow cytometry at the times indicated. Data are given as the mean and range of duplicate determinations, representative of three independent experiments.
involvement of AA and/or its metabolites in regulating cellular proliferation, other studies have reported the involvement of iPLA2 in cell growth via generation of AA, clearly indicating cell-type-specific differences. In a recent study, Herbert and Walker [38] described the involvement of iPLA2-VIA in the proliferative response of human umbilical endothelial cells to serum. Inhibition of iPLA2-VIA blocked proliferation, which could be partially restored by supplying the cell cultures with exogenous AA [38]. Similarly, work by Sánchez and Moreno [39] has attributed a key role for iPLA2-VIA-mediated AA release in regulating Caco-2 cell growth.

While our results have excluded that the eicosanoids have effects on cell-cycle progression, we cannot rule out the possibility that lysophospholipids generated by iPLA2-VIA could be involved in the response. As a matter of fact, iPLA2-VIA has been shown to mediate various responses of monocytes and U937 cells through lysophospholipid generation, namely secretion [10], various responses of monocytes and U937 cells through lysophospholipid generation, namel.

**Experimental procedures**

**Reagents**

[5,6,8,9,11,12,14,15-3H]AA (200 Ci mmol⁻¹) was purchased from Amersham Ibérica (Madrid, Spain). Methyl arachidonoyl fluorophosphonate (MAFP), bromoelanol lactone (BEL) and the human iPLA2-VIA antibody were purchased from Cayman Chemical (Ann Arbor, MI, USA). Pyrrophene was kindly provided by T. Ono (Shionogi Research Laboratories, Osaka, Japan). All other reagents were obtained from Sigma (St Louis, MO, USA).

**Cell culture**

U937 cells were kindly provided by P. Aller (Centro de Investigaciones Biológicas, Madrid, Spain). The cells were maintained in RPMI-1640 medium supplemented with 10% v/v fetal calf serum, 2 mM glutamine, penicillin (100 units mL⁻¹) and streptomycin (100 µg mL⁻¹) [47]. For experiments, the cells were incubated at 37°C in a humidified atmosphere of CO₂/air (1 : 19) at a cell density of 0.5–1 x 10⁶ cells mL⁻¹ in 12-well plastic culture dishes (Costar, Cambridge, MA, USA).

**PLA₂ activity assays**

For Ca²⁺-dependent PLA₂ activity, the mammalian membrane assay described by Diez et al. [48] was used. Briefly, aliquots of U937 cell homogenates were incubated for 1–2 h at 37°C in 100 mM Hepes (pH 7.5) containing 1.3 mM CaCl₂ and 100 000 d.p.m. of [³H]AA-labeled U937 cell membrane, used as substrate, in a final volume of 0.15 mL. Prior to assay, the cell membrane substrate was heated at 57°C for 5 min, in order to inactivate Ca-dependent transacylase activity [48]. The assay contained 25 µM LY311727 and 25 µM BEL to completely inhibit endogenous secreted and Ca²⁺-independent PLA₂ activities [30,49–51]. After lipid extraction, free [³H]arachidonic acid was separated by TLC using n-hexane/ethyl ether/acetic acid (70 : 30 : 1) as the mobile phase [52,53]. For Ca²⁺-independent PLA₂ activity, U937 cell aliquots were incubated for 2 h at 37°C in 100 mM Hepes (pH 7.5) containing 5 mM EDTA and 100 µM labeled phospholipid substrate (1-palmitoyl-2-[³H]palmitoyl-glycerol-3-phosphocholine, specific activity 60 Ci mmol⁻¹; American Radiolabeled Chemicals, St Louis, MO, USA) in a final volume of 150 µL. The phospholipid substrate was used in the form of sonicated vesicles in buffer. The reactions were quenched by adding 3.75 volumes of chloroform/methanol (1 : 2). After lipid extraction, free [³H]palmitic acid was separated by TLC using n-hexane/ethyl ether/acetic acid (70 : 30 : 1) as the mobile phase [52,53]. In some experiments, iPLA₂ activity was also measured utilizing a mixed-micelle substrate or the natural membrane assay. For the mixed-micelle assay, Triton X-100 was added to the dried
lipid substrate at a molar ratio of 4 : 1. Buffer was added and the mixed micelles were produced by a combination of heating above 40°C, vortexing and water bath sonication until the solution clarified. The natural membrane assay was carried out exactly as described above except that CaCl₂ was omitted and 5 mM EDTA was added instead. All of these assay conditions have been validated previously for U937 cell homogenates [18,25,54].

**Proliferation assay**

The CellTiter96 Aqueous One-Solution Cell Proliferation Assay (Promega Biotech Ibérica, Madrid, Spain) was used, following the manufacturer’s instructions. Briefly, cells (10 000 cells per well) were seeded in 96-well plates treated with vehicle or various concentrations of inhibitor. After 24 h, formazan product formation was assayed by recording absorbance at 490 nm using a 96-well plate reader.

**Antisense oligonucleotide treatments**

The iPLA₂-VIA antisense oligonucleotide utilized in this study has been described previously [24,25,27,55]. The anti- or sense oligonucleotides were mixed with lipofectamine, and complexes were allowed to form at room temperature for 10–15 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 trypan blue dye-exclusion assay.

**Immunoblot analyses**

Cells were lysed in ice-cold lysis buffer, and 15 μg of cellular protein from each sample were separated by standard 10% SDS-PAGE and transferred to nitrocellulose membranes. Primary and secondary antibodies were diluted in NaCl/Pi, containing 0.5% defatted dry milk and 0.1% Tween-20. After 1 h incubation with primary antibody at 1 : 1000, blots were washed three times and anti-rabbit secondary peroxidase-conjugated serum was added for another hour. Immunoblots were developed using the Amersham enhanced chemiluminescence system.

**Cell synchronization and cell-cycle analysis**

U937 cells were synchronized at G2/M by treating them with 0.05 μg/mL nocodazole for 12 h [32]. The cells were then washed, plated in fresh medium and allowed to progress through the cell cycle. After the indicated times, the cells were washed twice with cold NaCl/Pi, and fixed with 70% ethanol at 4°C for 18 h. Cells were then washed and resuspended in NaCl/Pi. RNA was removed by digestion with RNase A at room temperature. Staining was achieved by incubation with staining solution (500 μg/mL propidium iodide in NaCl/Pi) for 1 h, and cell-cycle analysis was performed by flow cytometry in a Coulter Epics XL-MCL cytofluorometer (Beckman Coulter, Fullerton, CA, USA).

**Quantification of the amount of PC**

Cell lipids were extracted using the Bligh and Dyer procedure [56], and the individual phospholipid species were fractionated by TLC in silica gel G plates using chloroform/methanol/acetic acid/water (65 : 501 : 4) as a solvent system [57]. The PC fraction was identified by comparison with commercial standards. The PC levels were quantified by measuring lipid phosphorus [58].

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**References**


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