the FEBS Journal

# Calcium-independent phospholipase $A_2$ mediates proliferation of human promonocytic U937 cells

María A. Balboa, Rebeca Pérez and Jesús Balsinde

Institute of Molecular Biology and Genetics, Spanish National Research Council (CSIC) and University of Valladolid School of Medicine, Spain

**Keywords**
cell cycle; human promonocytes; membrane phospholipid; phospholipase $A_2$; proliferation

**Correspondence**
J. Balsinde, Instituto de Biología y Genética Molecular, Calle Sanz y Forés s/n, 47003 Valladolid, Spain
Fax: +34 983 423 588
Tel: +34 983 423 062
E-mail: jbalsinde@ibgm.uva.es

(Received 26 December 2007, revised 15 February 2008, accepted 21 February 2008)

doi:10.1111/j.1742-4658.2008.06350.x

We have investigated the possible involvement of two intracellular phospholipases $A_2$, namely group VIA calcium-independent phospholipase $A_2$ (iPLA$_2$-VIA) and group IVA cytosolic phospholipase $A_2$ (cPLA$_2\alpha$), in the regulation of human promonocytic U937 cell proliferation. Inhibition of iPLA$_2$-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 cPLA$_2\alpha$ had no significant effect on U937 proliferation. Evaluation of iPLA$_2$-VIA activity in cell cycle-synchronized cells revealed highest activity at $G_2$/M and late S phases, and lowest at $G_1$. Phosphatidylcholine levels showed the opposite trend, peaking at $G_1$ and lowest at $G_2$/M and late S phase. Reduction of U937 cell proliferation by inhibition of iPLA$_2$-VIA activity was associated with arrest in $G_2$/M and S phases. The iPLA$_2$-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 phospholipase $A_2$ (PLA$_2$) 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 PLA$_2$s differ with respect to substrate specificity, co-factor requirements for activity, and cellular localization [1–5]. Mammalian cells usually contain several PLA$_2$s, and thus the challenge in recent years has been to ascribe specific cellular functions to particular PLA$_2$ forms. PLA$_2$s are systematically classified into several groups, many of which include various subgroups [5]. However, based on their biochemical commonalities, PLA$_2$s are usually grouped into four major families, namely $Ca^{2+}$-dependent secreted enzymes, $Ca^{2+}$-dependent cytosolic enzymes (cPLA$_2$), $Ca^{2+}$-independent cytosolic enzymes (iPLA$_2$), and platelet-activating factor acetyl hydrolases [1,5].

The iPLA$_2$ family consists of two members in mammalian cells, designated iPLA$_2$-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, iPLA$_2$-VIA has attracted considerable interest due to the multiple roles and functions that this enzyme may have in cells. Several splice variants of iPLA$_2$-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, iPLA$_2$-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 iPLA$_2$-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; cPLA$_2\alpha$, group IVA cytosolic phospholipase $A_2$; iPLA$_2$-VIA, group VIA calcium-independent phospholipase $A_2$; MAFP, methyl arachidonyl fluoromethyl phosphonate; PC, phosphatidylcholine; PLA$_2$, phospholipase $A_2$.

iPLA$_2$-VIA by treatment of cells with bromoenol lactone (BEL) [11,12]. In INS-1 insulinoma cells, acute inhibition of iPLA$_2$-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 iPLA$_2$-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 iPLA$_2$-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 iPLA$_2$-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 iPLA$_2$-VIA in the normal proliferative response of human promonocytic U937 cells, and compared it to that of another major intracellular PLA$_2$, the AA-selective cPLA$_2\alpha$. Utilizing various strategies, we demonstrate here that iPLA$_2$-VIA, but not cPLA$_2\alpha$, plays a key role in U937 cell proliferation by a mechanism that does not involve AA or one of its oxygenated metabolites.

## Results

### iPLA$_2$ inhibition slows U937 cell proliferation

Using RT-PCR, we have previously found that human promonocytic U937 cells express both cPLA$_2\alpha$ and iPLA$_2$-VIA, but, strikingly, do not express Group V, Group X or any of the group II secreted PLA$_2$s [24]. Enzymatic activities corresponding to both cPLA$_2\alpha$ and iPLA$_2$-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 PLA$_2$ inhibitors was examined. Figure 1 shows that the selective cPLA$_2\alpha$ inhibitor pyrrophenone [26] completely blocked the $Ca^{2+}$-dependent PLA$_2$ 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).

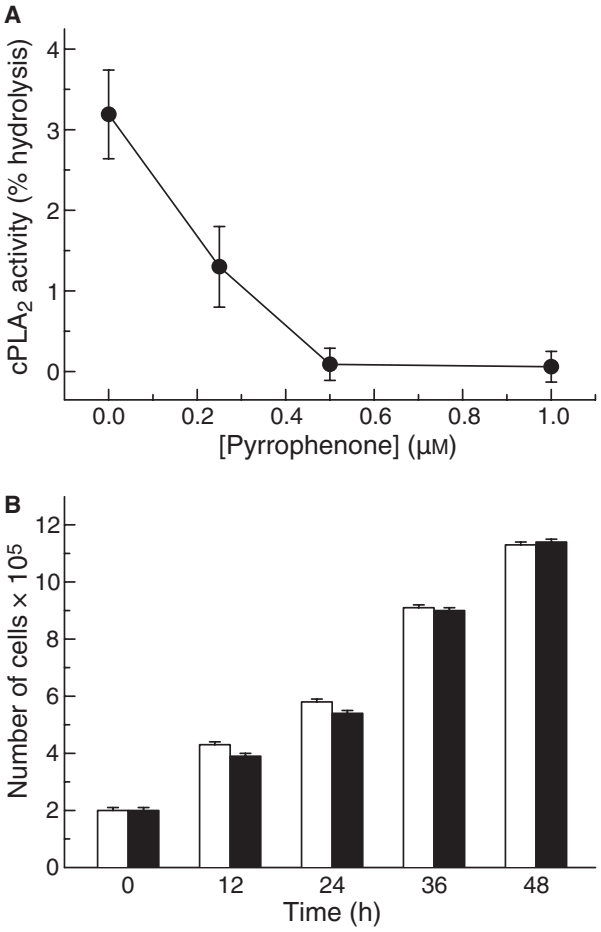


**Fig. 1.** Effect of pyrrophenone on the growth of U937 cells. (A) Dose–response curve for the effect of pyrrophenone on the $Ca^{2+}$-dependent PLA$_2$ activity of U937 cell homogenates. The cell membrane assay was utilized. (B) Time course of the effect of pyrrophenone on the proliferative capacity of U937 cells. The cells were incubated with (closed bars) or without (open bars) 1 μM pyrrophenone for the times indicated, and cell number was estimated as described in Experimental procedures. Data are given as means ± SEM of triplicate determinations, representative of three independent experiments.

In contrast to pyrrophenone, the iPLA$_2$ 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 iPLA$_2$ activity, as measured by an *in vitro* assay (Fig. 2). Collectively, the data in Figs 1 and 2 are consistent with the involvement of iPLA$_2$, but not cPLA$_2\alpha$, in U937 cell proliferation. Owing to the lack of specificity of BEL in cell-based assays [28], additional pharmacological evidence for the involvement of iPLA$_2$ in U937 cell growth was obtained using methyl

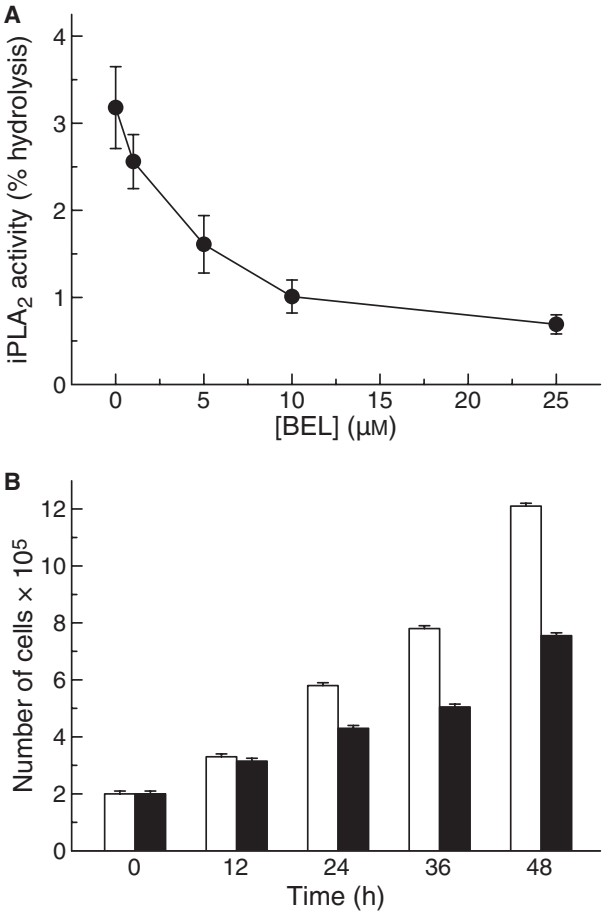


**Fig. 2.** Effect of BEL on the growth of U937 cells. (A) Dose–response curve for the effect of BEL on the $Ca^{2+}$-independent $PLA_2$ activity of U937 cell homogenates. The substrate was presented in the form of mixed micelles produced using Triton X-100. (B) Time course of the effect of BEL on the proliferative capacity of U937 cells. The cells were incubated with (closed bars) or without (open bars) 5 μM BEL for the times indicated, and cell number was estimated as described in Experimental procedures. Data are given as means ± SEM of triplicate determinations, representative of three independent experiments.

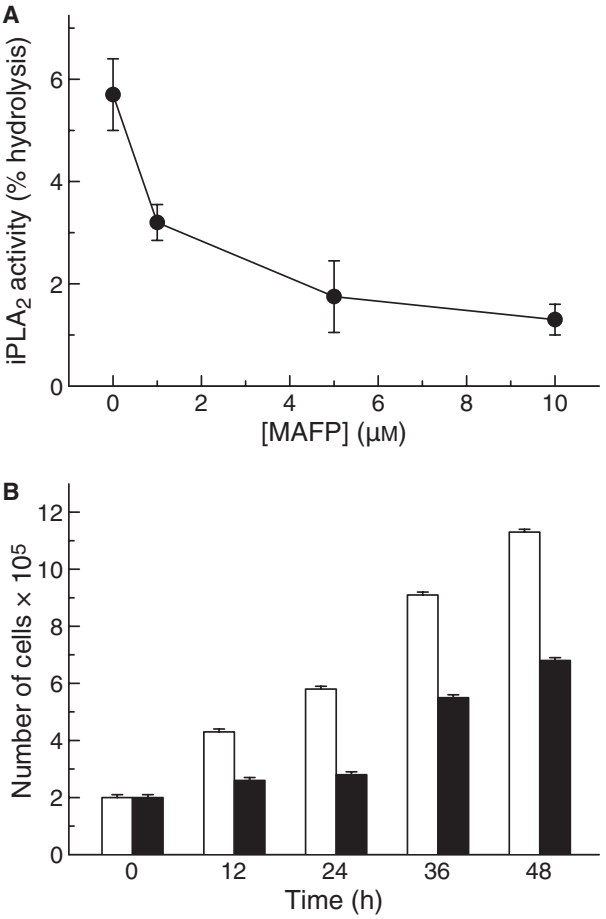


**Fig. 3.** Effect of MAFP on the growth of U937 cells. (A) Dose–response curve for the effect of MAFP on the $Ca^{2+}$-independent $PLA_2$ activity of U937 cell homogenates. The substrate was presented in the form of mixed micelles produced using Triton X-100. (B) Time course of the effect of MAFP on the proliferative capacity of U937 cells. The cells were incubated with (closed bars) or without (open bars) 10 μM MAFP for the times indicated, and cell number was estimated as described in Experimental procedures. Data are given as means ± SEM of triplicate determinations, representative of three independent experiments.

arachidonyl fluoromethyl phosphonate (MAFP), a dual iPLA$_2$/cPLA$_2$ inhibitor that is structurally unrelated to BEL and pyrrophenone [30,31]. Concentrations of MAFP that completely inhibited cellular $Ca^{2+}$-independent $PLA_2$ activity also led to strong inhibition of U937 cell growth (Fig. 3). Given that the pyrrophenone experiments had established that cPLA$_2\alpha$ is not critical for U937 cell growth, the inhibitory effect of MAFP seen in Fig. 3 can be attributed to inhibition of iPLA$_2$.

To confirm iPLA$_2$ involvement in U937 cell growth in a more definitive manner, the effect of an antisense oligonucleotide directed against iPLA$_2$-VIA was evaluated. In these experiments, the antisense construct produced a 70–75% decrease in immunoreactive iPLA$_2$-VIA and markedly inhibited (30–40%) U937 cell proliferation (Fig. 4).

## Inhibition of iPLA$_2$ does not induce cell death

Trypan blue assays after the various treatments leading to iPLA$_2$ inhibition indicated no loss of viability, suggesting that necrotic cell death did not occur. To examine the possibility of apoptotic cell death, we

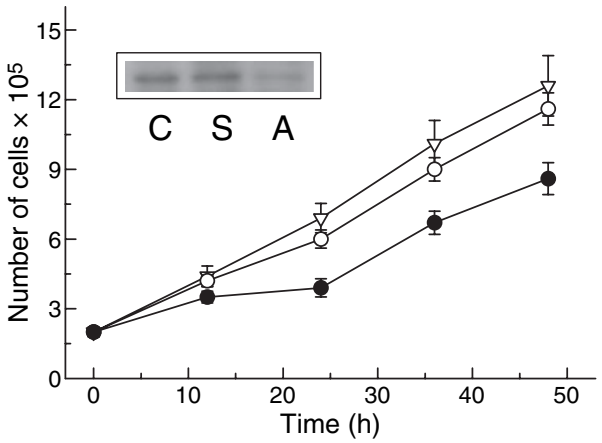


**Fig. 4.** Antisense inhibition of iPLA$_2$-VIA slows the growth of U937 cells. The cells were either untreated (inverted triangles), or treated with sense (open circles) or antisense (closed circles) oligonucleotides, and cell number was estimated as described in Experimental procedures. The inset shows the iPLA$_2$-VIA protein level after the various treatments (C, control cells; S, sense-treated cells; A, antisense-treated cells), as analyzed by immunoblot. Data are given as the mean and range of duplicate determinations, representative of five independent experiments.

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 iPLA$_2$ 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 iPLA$_2$-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 iPLA$_2$-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 iPLA$_2$ activity by either pharmacological or antisense methods arises as a result of slowed cell division and not increased apoptosis.

## iPLA$_2$ activity during the cell cycle

To obtain more information on the role of iPLA$_2$ on U937 cell growth, we used flow cytometry to examine the cell-cycle dependence of iPLA$_2$ 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

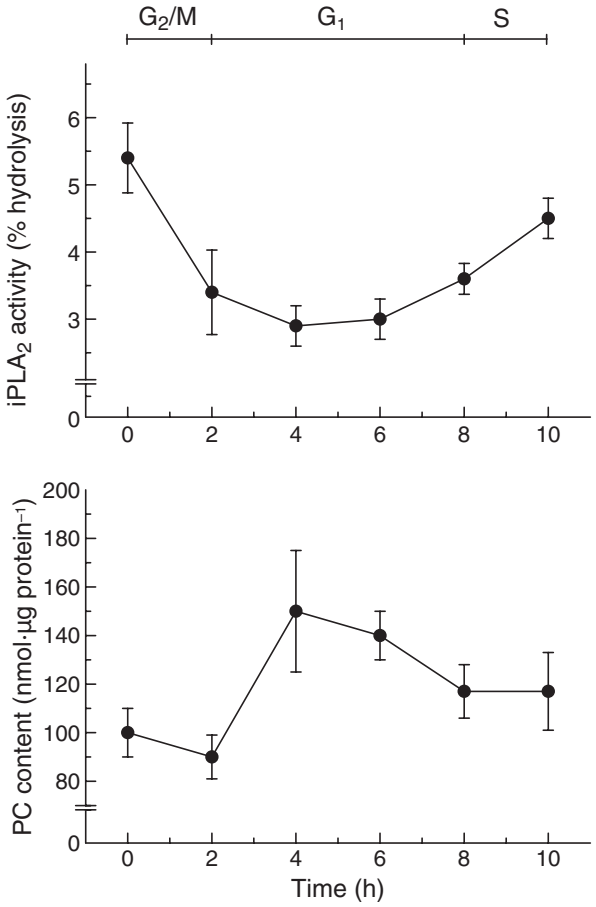


**Fig. 5.** Changes in iPLA$_2$ activity and PC content during the cell cycle. The cells were synchronized with nocodazole as described in Experimental procedures. iPLA$_2$ activity and PC content were measured at various times after releasing the cells from the nocodazole block, as indicated. Data are given as mean ± SE of triplicate determinations, representative of five independent experiments.

after release from the mitotic block with nocodazole, more than 75% of the cells were in the G$_2$/M phase (Fig. 5). The cells were in G$_1$ 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 G$_2$/M throughout the G$_1$ and S phases [23,32].

iPLA$_2$ activity measurements during the cell cycle revealed significant differences depending on the phase (Fig. 5). Highest activity was found during G$_2$/M, decreasing as the cells entered G$_1$ and then increasing again as the cells approached and entered S phase. The same pattern of variation of iPLA$_2$ 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 iPLA$_2$ activity (Fig. 5). PC levels rose abruptly in early $G_1$ and then slowly declined as the cells progressed into late $G_1$ and S (Fig. 5). That PC levels and iPLA$_2$ activity show opposite kinetics is fully consistent with the possibility that iPLA$_2$ behaves as a major regulator of PC catabolism, which is responsible for glycerophospholipid accumulation during the cell cycle [23,33]. Thus, decreased iPLA$_2$ activity during the $G_1$ phase would result in an increase in PC content due to reduced catabolism.

### Induction of cell cycle arrest by iPLA$_2$ inhibition

Having established that iPLA$_2$ 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 iPLA$_2$ inhibition was a consequence of cell-cycle arrest. The cells were synchronized with nocodazole and then treated with BEL to inhibit iPLA$_2$ 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 $G_2$/M and S, and a concomitant decrease of cells in $G_1$, 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 iPLA$_2$, but not cPLA$_2\alpha$, causes cell arrest in S and $G_2$/M phases.

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

In addition to its role in PC homeostasis, iPLA$_2$, 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 iPLA$_2$ by BEL or antisense technology. However, the results indicated that AA failed to restore the growth of cells deficient in iPLA$_2$ 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 iPLA$_2$ on U937 cell proliferation.

## Discussion

In this study, we demonstrate that iPLA$_2$-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 iPLA$_2$-VIA by either pharmacological means or antisense technology slows growth by inducing arrest at the S and $G_2$/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 iPLA$_2$-VIA is not mediated via AA-derived mitogenic signaling. We also show that U937 cell iPLA$_2$-VIA activity is regulated in a cell-cycle-dependent manner, with maximal activity at $G_2$/M, steadily declining during $G_1$, 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 $G_1$. This inverse relationship between the kinetics of iPLA$_2$-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 iPLA$_2$-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 iPLA$_2$-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 iPLA$_2$-VIA activity in $G_1$ and early S phase would allow accumulation of phospholipid in preparation for future cell division. Once cells enter S phase, the level of iPLA$_2$-VIA begins to increase, which would slow down phospholipid accumulation. It is interesting to note, however, that iPLA$_2$-VIA might not always be the major regulator of phospholipid catab-

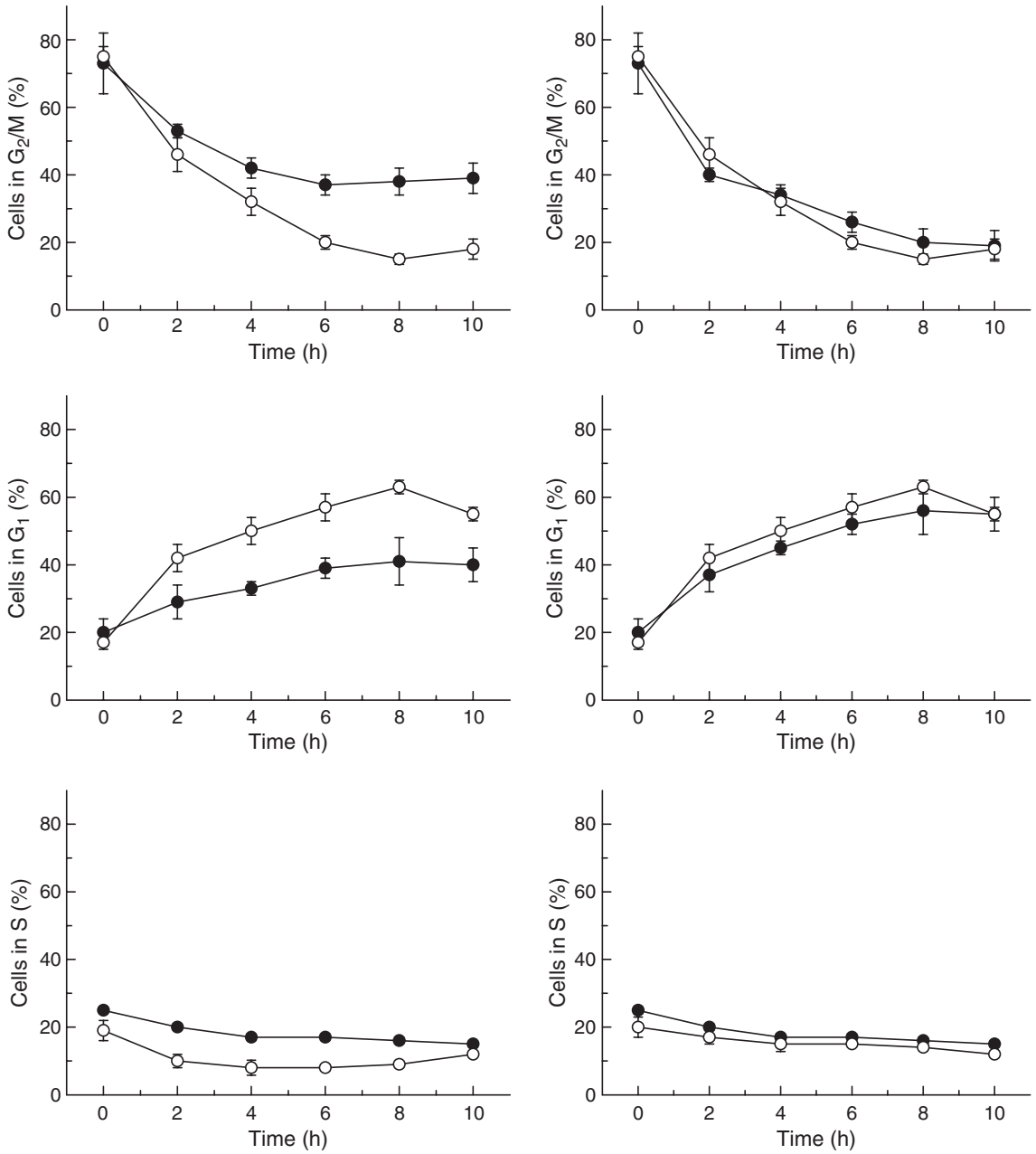


**Fig. 6.** Effect of BEL and pyrrophenone on the U937 cell cycle. The cells were synchronized with nocodazole as described in Experimental procedures. After releasing the cells from the nocodazole block, they were untreated (open symbols) or treated with 5 μM BEL (closed symbols, left column) or 1 μM 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.

olism during cell cycle progression. Our data suggest that, 2 h after cell cycle entry, iPLA$_2$ 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, iPLA$_2$-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

involvement of AA and/or its metabolites in regulating cellular proliferation, other studies have reported the involvement of iPLA$_2$ 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 iPLA$_2$-VIA in the proliferative response of human umbilical endothelial cells to serum. Inhibition of iPLA$_2$-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 iPLA$_2$-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 iPLA$_2$-VIA could be involved in the response. As a matter of fact, iPLA$_2$-VIA has been shown to mediate various responses of monocytes and U937 cells through lysophospholipid generation, namely secretion [10], apoptosis [24,40,41] and possibly chemotaxis [42,43].

The involvement of specific PLA$_2$ forms in the regulation of cell division may also be a cell-type-specific event. Although our results did not implicate cPLA$_2\alpha$ – a well-established signal-activated enzyme [34] – in regulating cellular proliferation, other studies have reported the involvement of this enzyme. In the aforementioned system of human umbilical endothelial cells, the importance of cPLA$_2\alpha$-mediated AA release in the regulation of cell proliferation was also documented [44]. Thus the suggestion was made that both enzymes may somehow cooperate in regulating endothelial cell proliferation via generation of AA [38,44]. In contrast, the work by Sánchez and Moreno [39] attributed a key role for iPLA$_2$-VIA-mediated AA release in regulating Caco-2 cell growth (as mentioned above), but ruled out a role for cPLA$_2\alpha$ in the process. However, studies in vascular smooth muscle cells by Anderson *et al.* [45] highlighted the very important role of cPLA$_2\alpha$ in the process but a lack of involvement of iPLA$_2$-VIA. Importantly, in a recent study with neuroblastoma cells, van Rossum *et al.* [46] demonstrated the involvement of cPLA$_2\alpha$ in cell-cycle progression, and although a role for iPLA$_2$ in this system was not ascertained, the observation was made that redundancy of functions between cPLA$_2$ and iPLA$_2$ may exist under certain conditions. We are currently performing experiments with various cell systems to study the possibility of cross-talk between cPLA$_2\alpha$ and iPLA$_2$-VIA in cell proliferation, and also to define whether the role of iPLA$_2$-VIA in cell growth is to directly generate growth factor-like lipids and/or to regulate changes in overall phospholipid metabolism that could trigger the activation *in situ* of intracellular mitogenic pathways.

## Experimental procedures

### Reagents

[5,6,8,9,11,12,14,15-$^3$H]AA (200 Ci·mmol$^{-1}$) was purchased from Amersham Ibérica (Madrid, Spain). Methyl arachidonyl fluorophosphonate (MAFP), bromoenol lactone (BEL) and the human iPLA$_2$-VIA antibody were purchased from Cayman Chemical (Ann Arbor, MI, USA). Pyrrophenone 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 $CO_2$/air (1 : 19) at a cell density of 0.5–1 × 10$^6$ cells·mL$^{-1}$ in 12-well plastic culture dishes (Costar, Cambridge, MA, USA).

### PLA$_2$ activity assays

For $Ca^{2+}$-dependent PLA$_2$ 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 $CaCl_2$ and 100 000 d.p.m. of [$^3$H]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 CoA-independent transacylase activity [48]. The assay contained 25 μM LY311727 and 25 μM BEL to completely inhibit endogenous secreted and $Ca^{2+}$-independent PLA$_2$ activities [30,49–51]. After lipid extraction, free [$^3$H]arachidonic acid was separated by TLC using *n*-hexane/ethyl ether/acetic acid (70 : 30 : 1) as the mobile phase [52,53]. For $Ca^{2+}$-independent PLA$_2$ 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-[$^3$H]palmitoyl-glycero-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 [$^3$H]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, iPLA$_2$ 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 $CaCl_2$ 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 iPLA$_2$-VIA antisense oligonucleotide utilized in this study has been described previously [24,25,27,55]. The antisense 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 $\mu g \cdot mL^{-1}$, 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/$P_i$ 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 $G_2$/M by treating them with 0.05 $\mu g \cdot mL^{-1}$ 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/$P_i$, and fixed with 70% ethanol at 4°C for 18 h. Cells were then washed and resuspended in NaCl/$P_i$. RNA was removed by digestion with RNase A at room temperature. Staining was achieved by incubation with staining solution (500 $\mu g \cdot mL^{-1}$ propidium iodide in NaCl/$P_i$) 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].

## Acknowledgements

We thank Montse Duque and Yolanda Sáez for expert technical assistance. This work was supported by the Spanish Ministry of Education and Science (grant nos BFU2004-01886/BMC, SAF2004-04676, BFU2007-67154/BMC and SAF2007-60055) and the Fundación La Caixa (grant no. BM05-248-0).

## References

1 Balsinde J, Winstead MV & Dennis EA (2002) Phospholipase $A_2$ regulation of arachidonic acid mobilization. *FEBS Lett* **531**, 2–6.
2 Balboa MA, Varela-Nieto I, Lucas KK & Dennis EA (2002) Expression and function of phospholipase $A_2$ in brain. *FEBS Lett* **531**, 12–17.
3 Balsinde J & Balboa MA (2005) Cellular regulation and proposed biological functions of group VIA calcium-independent phospholipase $A_2$ in activated cells. *Cell Signal* **17**, 1052–1062.
4 Balboa MA & Balsinde J (2006) Oxidative stress and arachidonic acid mobilization. *Biochim Biophys Acta* **1761**, 385–391.
5 Schaloske R & Dennis EA (2006) The phospholipase $A_2$ superfamily and its group numbering system. *Biochim Biophys Acta* **1761**, 1246–1259.
6 Balsinde J & Dennis EA (1997) Function and inhibition of intracellular calcium-independent phospholipase $A_2$. *J Biol Chem* **272**, 16069–16072.
7 Winstead MV, Balsinde J & Dennis EA (2000) Calcium-independent phospholipase $A_2$. Structure and function. *Biochim Biophys Acta* **1488**, 28–39.
8 Ackermann EJ, Kempner ES & Dennis EA (1994) $Ca^{2+}$-independent cytosolic phospholipase $A_2$ from macrophage-like P388$D_1$ cells. Isolation and characterization. *J Biol Chem* **269**, 9227–9233.

9 Tang J, Kriz RW, Wolfman N, Shaffer M, Seehra J & Jones SS (1997) A novel cytosolic calcium-independent phospholipase $A_2$ contains eight ankyrin motifs. *J Biol Chem* **272**, 8567–8580.

10 Balboa MA, Balsinde J, Jones SS & Dennis EA (1997) Identity between the $Ca^{2+}$-independent phospholipase $A_2$ enzymes from P388$D_1$ macrophages and Chinese hamster ovary cells. *J Biol Chem* **272**, 8576–8580.

11 Balsinde J, Barbour SE, Bianco ID & Dennis EA (1994) Arachidonic acid mobilization in P388$D_1$ macrophages is controlled by two distinct $Ca^{2+}$-dependent phospholipase $A_2$ enzymes. *Proc Natl Acad Sci USA* **91**, 11060–11064.

12 Ramanadham S, Hsu FF, Bohrer A, Ma Z & Turk J (1999) Studies of the role of group VI phospholipase $A_2$ in fatty acid incorporation, phospholipid remodeling, lysophosphatidylcholine generation, and secretagogue-induced arachidonic acid release in pancreatic islets and insulinoma cells. *J Biol Chem* **274**, 13915–13927.

13 Balsinde J, Balboa MA & Dennis EA (1997) Antisense inhibition of group VI $Ca^{2+}$-independent phospholipase $A_2$ blocks phospholipid fatty acid remodeling in murine P388$D_1$ macrophages. *J Biol Chem* **272**, 29317–29321.

14 Balboa MA, Sáez Y & Balsinde J (2003) Calcium-independent phospholipase $A_2$ is required for lysozyme secretion in U937 promonocytes. *J Immunol* **170**, 5276–5280.

15 Daniele JJ, Fidelio GD & Bianco ID (1999) Calcium dependency of arachidonic acid incorporation into cellular phospholipids of different cell types. *Prostaglandins* **57**, 341–350.

16 Alzola E, Perez-Etxebarria A, Kabre E, Fogarty DJ, Metioui M, Chaib N, Macarulla JM, Matute C, Dehaye JP & Marino A (1998) Activation by P2X7 agonists of two phospholipases $A_2$ ($PLA_2$) in ductal cells of rat submandibular gland. Coupling of the calcium-independent PLA2 with kallikrein secretion. *J Biol Chem* **273**, 30208–30217.

17 Birbes H, Drevet S, Pageaux JF, Lagarde M & Laugier C (2000) Involvement of calcium-independent phospholipase $A_2$ in uterine stromal cell phospholipid remodeling. *Eur J Biochem* **267**, 7118–7127.

18 Balsinde J (2002) Roles of various phospholipases $A_2$ in providing lysophospholipid acceptors for fatty acid phospholipid incorporation and remodelling. *Biochem J* **364**, 695–702.

19 Pérez R, Melero R, Balboa MA & Balsinde J (2004) Role of group VIA calcium-independent phospholipase $A_2$ in arachidonic acid release, phospholipid fatty acid incorporation, and apoptosis in U937 cells responding to hydrogen peroxide. *J Biol Chem* **279**, 40385–40391.

20 Baburina I & Jackowski S (1999) Cellular responses to excess phospholipid. *J Biol Chem* **274**, 9400–9408.

21 Barbour SE, Kapur A & Deal CL (1999) Regulation of phosphatidylcholine homeostasis by calcium-independent phospholipase $A_2$. *Biochim Biophys Acta* **1439**, 77–88.

22 Walkey CJ, Kalmar GB & Cornell RB (1994) Overexpression of rat liver CTP:phosphocholine cytidylyltransferase accelerates phosphatidylcholine synthesis and degradation. *J Biol Chem* **269**, 5742–5749.

23 Manguikian AD & Barbour SE (2004) Cell cycle dependence of group VIA calcium-independent phospholipase $A_2$ activity. *J Biol Chem* **279**, 52881–52892.

24 Pérez R, Balboa MA & Balsinde J (2006) Involvement of group VIA calcium-independent phospholipase $A_2$ in macrophage engulfment of hydrogen peroxide-treated U937 cells. *J Immunol* **176**, 2555–2561.

25 Balboa MA & Balsinde J (2002) Involvement of calcium-independent phospholipase $A_2$ in hydrogen peroxide-induced accumulation of free fatty acids in human U937 cells. *J Biol Chem* **277**, 40384–40389.

26 Ono T, Yamada K, Chikazawa Y, Ueno M, Nakamoto S, Okuno T & Seno K (2002) Characterization of a novel inhibitor of cytosolic phospholipase $A_2\alpha$. *Biochem J* **363**, 727–735.

27 Fuentes L, Pérez R, Nieto ML, Balsinde J & Balboa MA (2003) Bromoenol lactone promotes cell death by a mechanism involving phosphatidate phosphohydrolase-1 rather than calcium-independent phospholipase $A_2$. *J Biol Chem* **278**, 44683–44690.

28 Balsinde J, Pérez R & Balboa MA (2006) Calcium-independent phospholipase $A_2$ and apoptosis. *Biochim Biophys Acta* **1761**, 1344–1350.

29 Pindado J, Balsinde J & Balboa MA (2007) TLR3-dependent induction of nitric oxide synthase in RAW 264.7 macrophage-like cells via a cytosolic phospholipase $A_2$/cyclooxygenase-2 pathway. *J Immunol* **179**, 4821–4828.

30 Balsinde J & Dennis EA (1996) Distinct roles in signal transduction for each of the phospholipase $A_2$ enzymes present in P388$D_1$ macrophages. *J Biol Chem* **271**, 6758–6765.

31 Lio YC, Reynolds LJ, Balsinde J & Dennis EA (1996) Irreversible inhibition of $Ca^{2+}$-independent phospholipase $A_2$ by methyl arachidonyl fluorophosphonate. *Biochim Biophys Acta* **1302**, 55–60.

32 Roshak AK, Capper EA, Stevenson C, Eichman C & Marshall LA (2000) Human calcium-independent phospholipase $A_2$ mediates lymphocyte proliferation. *J Biol Chem* **275**, 35692–35698.

33 Jackowski S (1996) Cell cycle regulation of membrane phospholipid metabolism. *J Biol Chem* **271**, 20219–20222.

34 Nakanishi M & Rosenberg DW (2006) Roles of $cPLA_2\alpha$ and arachidonic acid in cancer. *Biochim Biophys Acta* **1761**, 1335–1343.

35 Golfman LS, Bakovic M & Vance DE (2001) Transcription of the CTP:phosphocholine cytidylyltransferase alpha gene is enhanced during the S phase of the cell cycle. *J Biol Chem* **276**, 43688–43692.
36 Song Y, Wilkins P, Hu W, Murthy KS, Chen J, Lee Z, Oyesanya R, Wu J, Barbour SE & Fang X (2007) Inhibition of calcium-independent phospholipase $A_2$ suppresses proliferation and tumorigenicity of ovarian carcinoma cells. *Biochem J* **406**, 427–436.
37 Zhang XH, Zhao C, Seleznev K, Song K, Manfredi JJ & Ma ZA (2006) Disruption of $G_1$-phase phospholipid turnover by inhibition of $Ca^{2+}$-independent phospholipase $A_2$ induces a p53-dependent cell-cycle arrest in $G_1$ phase. *J Cell Sci* **119**, 1005–1015.
38 Herbert SP & Walker JH (2006) Group VIA calcium-independent phospholipase $A_2$ mediates endothelial cell progression. *J Biol Chem* **281**, 35709–35716.
39 Sánchez T & Moreno JJ (2002) Calcium-independent phospholipase $A_2$ through arachidonic acid mobilization is involved in Caco-2 cell growth. *J Cell Physiol* **193**, 293–298.
40 Lauber K, Bohn E, Krober SM, Xiao YJ, Blumenthal SG, Lindemann RK, Marini P, Wiedig C, Zobywalski A, Baksh S *et al.* (2003) Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell* **113**, 717–730.
41 Kim SJ, Gershov D, Ma X, Brot N & Elkon KB (2002) I-PLA$_2$ activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine leading to binding by natural immunoglobulin M antibodies and complement activation. *J Exp Med* **196**, 655–665.
42 Carnevale KA & Cathcart MK (2001) Calcium-independent phospholipase $A_2$ is required for human monocyte chemotaxis to monocyte chemoattractant protein 1. *J Immunol* **167**, 3414–3421.
43 Mishra RS, Carnevale KA & Cathcart MK (2008) iPLA$_2\beta$: front and center in human monocyte chemotaxis to MCP-1. *J Exp Med* **205**, 347–359.
44 Herbert SP, Ponnambalam S & Walker JH (2005) Cytosolic phospholipase $A_2\alpha$ mediates endothelial cell proliferation and is inactivated by association with the Golgi apparatus. *Mol Biol Cell* **16**, 3800–3809.
45 Anderson KA, Roshak A, Winkler JD, McCord M & Marshall LA (1997) Cytosolic 85-kDa phospholipase $A_2$-mediated release of arachidonic acid is critical for proliferation of vascular smooth muscle cells. *J Biol Chem* **272**, 30504–30511.
46 van Rossum GSAT, Bijvelt JJM, van den Bosch H, Verkleij AJ & Boonstra J (2002) Cytosolic phospholipase $A_2$ and lipoxygenase are involved in cell cycle progression in neuroblastoma cells. *Cell Mol Life Sci* **59**, 181–188.
47 Balsinde J & Mollinedo F (1991) Platelet-activating factor synergizes with phorbol myristate acetate in activating phospholipase D in the human promonocytic cell line U937. Evidence for different mechanisms of activation. *J Biol Chem* **266**, 18726–18730.
48 Diez E, Chilton FH, Stroup G, Mayer RJ, Winkler JD & Fonteh AN (1994) Fatty acid and phospholipid selectivity of different phospholipase $A_2$ enzymes studied by using a mammalian membrane as substrate. *Biochem J* **301**, 721–726.
49 Schevitz RW, Bach NJ, Carlson DG, Chirgadze NY, Clawson DK, Dillard RD, Draheim SE, Hartley LW, Jones ND, Mihelich ED *et al.* (1995) Structure-based design of the first potent and selective inhibitor of human non-pancreatic secretory phospholipase $A_2$. *Nat Struct Biol* **2**, 458–465.
50 Hazen SL, Zupan LA, Weiss RH, Getman DP & Gross RW (1991) Suicide inhibition of canine myocardial cytosolic calcium-independent phospholipase $A_2$. Mechanism-based discrimination between calcium-dependent and -independent phospholipases A2. *J Biol Chem* **266**, 7227–7232.
51 Balsinde J, Balboa MA, Insel PA & Dennis EA (1999) Regulation and inhibition of phospholipase $A_2$. *Annu Rev Pharmacol Toxicol* **39**, 175–189.
52 Diez E, Balsinde J, Aracil M & Schüller A (1987) Ethanol induces release of arachidonic acid but not synthesis of eicosanoids in mouse peritoneal macrophages. *Biochim Biophys Acta* **921**, 82–89.
53 Balsinde J, Fernández B, Solís-Herruzo JA & Diez E (1992) Pathways for arachidonic acid mobilization in zymosan-stimulated mouse peritoneal macrophages. *Biochim Biophys Acta* **1136**, 75–82.
54 Balboa MA, Pérez R & Balsinde J (2003) Amplification mechanisms of inflammation: paracrine stimulation of arachidonic acid mobilization by secreted phospholipase $A_2$ is regulated by cytosolic phospholipase $A_2$-derived hydroperoxyeicosatetraenoic acid. *J Immunol* **171**, 989–994.
55 Pérez R, Matabosch X, Llebaria A, Balboa MA & Balsinde J (2006) Blockade of arachidonic acid incorporation into phospholipids induces apoptosis in U937 promonocytic cells. *J Lipid Res* **47**, 484–491.
56 Bligh EG & Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911–917.
57 Balsinde J, Diez E, Schüller A & Mollinedo F (1988) Phospholipase $A_2$ activity in resting and activated human neutrophils. Substrate specificity, pH dependence, and subcellular localization. *J Biol Chem* **263**, 1929–1936.
58 Rouser G, Fleischer S & Yamamoto A (1970) Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **8**, 494–496.