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The effect of high molecular phospholipase A_2 inhibitors on 3T6 fibroblast proliferation

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Abstract

Recently, we suggested that arachidonic acid and/or its cyclooxygenase pathway metabolites may be involved in regulating 3T6 fibroblast proliferation. In the present study we evaluate the role of high-molecular phospholipase A_2 (PLA_2) enzymes in the 3T6 fibroblast growth. Our results demonstrate that the cytosolic PLA_2 inhibitor, arachidonyl trifluoromethylketone and the cytosolic calcium-independent PLA_2 ($iPLA_2$) inhibitor, bromoenol lactone, decrease arachidonic acid release and prostaglandin E_2 production in 3T6 fibroblast cultures stimulated by fetal calf serum. These effects were correlated with the impairment of 3T6 fibroblast proliferation and DNA synthesis at the S/G_2 boundary, which prolongs the S phase. These data suggest a role of $iPLA_2$ in the control of 3T6 fibroblast growth.

Keywords: Cell growth; Phospholipase A_2; Arachidonic acid; Prostaglandins; Bromoenol lactone; Arachidonyl trifluoromethylketone

1. Introduction

Considerable amounts of arachidonic acid are found esterified in the membranes of mammalian cells and later released via phospholipase A_2 (E.C. 3.1.1.4, PLA_2 hydrolysis of the acyl bond at the sn-2 position [1]. Then, free intracellular arachidonic acid (AA) can be metabolized by prostaglandin H endoperoxide synthases (cyclooxygenases) [2] to produce prostanoids, the predominant AA metabolites synthesized by fibroblasts [3].

PLA_2 comprises a large superfamily of distinct enzymes that exhibit different substrate specificity, cofactor requirement, and subcellular localization [4]. Type II (14-kDa), a secreted PLA_2 form is well characterized and known to exist in both an extracellular form in inflammatory fluids [5] and in a cell-associated form [6]. The cytosolic 85-kDa PLA_2 ($cPLA_2$) is structurally distinct and, unlike the 14-kDa PLA_2, shows a preference for AA in the sn-2 position of cellular phospholipids and is regulated by physiological intracellular calcium concentrations and phosphorylation [7,8]. Recently, an 80-kDa calcium-independent cytosolic PLA_2 ($iPLA_2$) was identified in macrophages. This enzyme shares some characteristics with secreted PLA_2 and others with $cPLA_2$ and may serve as a housekeeping enzyme involved in the remodeling of membrane phospholipids [9]. These enzymes are induced by inflammatory cytokines and growth factors, and they influence cellular AA release and the subsequent eicosanoid production [10]. However, their contribution to the regulation of cell proliferation has not been examined.

The AA metabolites appear to have an important but yet undefined role in growth-dependent signaling pathways. Moreover, AA and eicosanoids serve as intermediates in growth factor signaling pathways, and AA and eicosanoids are effectors and targets for second-messenger interaction [11]. Furthermore, most tumor cells produce AA metabolites, which modulate a wide range of biological effects that induce growth and invasiveness of tumors [12]. Thus, we recently suggested that AA and/or its cyclooxygenase pathway metabolites may be involved in regulating cellular proliferation of 3T6 fibroblasts [13–15]. The present study attempted to evaluate the role of high-molecular PLA_2 enzyme inhibitors in 3T6 fibroblast proliferation. Our results demonstrate that a $cPLA_2$ and/or $iPLA_2$ inhibitors decreases

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Abbreviations: AA, arachidonic acid; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; $iPLA_2$, calcium-independent phospholipase A_2; FCS, fetal calf serum; BEL, bromoenol lactone; $AACOCF_3$, arachidonyl trifluoromethylketone; PGE_2, prostaglandin E_2

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AA release and prostaglandin E_2 production in 3T6 fibroblast cultures. These effects were correlated with the impairment of DNA synthesis and 3T6 growth stimulated by fetal calf serum.

2. Materials and methods

2.1. Materials

RPMI 1640, fetal calf serum (FCS), penicillin G, streptomycin and trypsin/EDTA were purchased from Life Technologies. [5,6,8,9,11,12,14,15-^{3}H]AA (180–240 Ci/mmol) and [*methyl*-^{3}H]thymidine (20 Ci/mmol) were obtained from Du Pont-New England Nuclear. Propidium iodide, RNase and manoalide were supplied by Sigma Chemical Co. Bromoenol lactone (BEL), an $iPLA_2$ inhibitor, and arachidonyl trifluoromethylketone ($AACOCF_3$), an non-selective inhibitor of high molecular weight PLA_2, were acquired from Alexis Corp. Antibodies directed against $cPLA_2$ and $iPLA_2$ were from Santa Cruz Biotechnology Inc. and Cayman Chemicals Co., respectively. Flosulide, an cyclooxygenase-2 inhibitor, was kindly provided by Dr. J. Queralt. All other reagents were of analytical grade.

2.2. Cell culture

Murine 3T6 fibroblasts (ATCC CCL96) were grown and maintained as previously described [15]. Thus, fibroblasts were grown in RPMI 1640 containing 10% FCS, and penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were harvested with trypsin/EDTA and passed to tissue-culture plates with a surface area of 5 cm^2/well (tissue-culture cluster 12; Costar). Cell cultures were maintained in a temperature- and humidity-controlled incubator at 37° with 95% air-5% CO_2. Cell viability tests were performed using the trypan blue exclusion test.

2.3. SDS-polyacrylamide gel electrophoresis/immunoblotting

Proteins from cell lysates (10^5 cells) were resolved by electrophoresis on SDS-PAGE (7.5%) gels under reducing conditions. The separated proteins were electroblotted onto nitrocellulose membranes using a Miniprotean II system (BioRad), according to the manufacturer's instructions. The membranes were probed with $cPLA_2$ and $iPLA_2$ antibodies (1:2000) and visualized using the ECL Western blot analysis system (Amersham Pharmacia, Biotech).

2.4. Incorporation and release of [^{3}H]AA

After a period of fibroblast replication (3–4 days) and a period of FCS starvation (6 hr), the medium was removed and replaced with 0.5 mL RPMI 1640 containing 0.1% fatty acid-free BSA and 0.1 μCi [^{3}H]AA for 24 hr. [^{3}H]AA incorporation was performed in preconfluent cultures (1000–3000 cells/cm^2.). Cells were then washed three times with medium containing 0.5% BSA to remove unincorporated [^{3}H]AA. After a study period, the medium was removed for analysis of radioactivity released. The amount of [^{3}H]AA released into the medium was expressed as a percentage of cell-incorporated [^{3}H]AA, which was determined in solubilized cells. Background release from untreated cells (8 $\pm$ 1% of [^{3}H]AA incorporated) was subtracted from all data.

2.5. Measurement of prostaglandin E_2 levels

An aliquot of culture medium (0.25 mL) was acidified with 1 mL of 1% formic acid. Prostaglandin E_2 was extracted in ethyl acetate (5 mL), and, after the aqueous phase was discarded, the organic phase was evaporated under a stream of nitrogen. The overall recovery for the extraction procedure was established by including [^{3}H]PGE_2 and was found to be 87 $\pm$ 2%. PGE_2 levels were determined using a PGE_2-monoclonal enzyme immunoassay kit (Cayman Chemicals Co.), following the manufacturer's protocol.

2.6. Cell growth

The influence of treatments was assessed on 3T6 fibroblasts plated at 3000 cells/well in 12-well plates and cultured for 3 days in RPMI 1640-supplemented with 10% FCS in presence of different treatments. Finally, the cells were washed, trypsinized, and counted. Medium plus treatments were changed each day to avoid the possible breakdown of the drugs.

2.7. Analysis of DNA synthesis

DNA synthesis was measured by a [^{3}H]thymidine incorporation assay. This involved culturing 3T6 fibroblasts in 96-well plates (Costar) in RPMI 1640 with 10% FCS at a density of 400 cells/well. Six hours later, cells were incubated with the drugs and [^{3}H]thymidine (1 μCi/well) for 24 hr. Then, [^{3}H]thymidine-containing media were aspirated, and cells were washed three times with medium containing 0.5% BSA to remove unincorporated [^{3}H]thymidine. Finally, cells were overlaid with 1% Triton X-100, and then cells were scraped off the dishes. Finally, radioactivity present in the cell fraction was measured by scintillation counting, using a Packard Tri-Carb 1500 counter.

2.8. Cell cycle analysis

For analysis of cell cycle distribution, treated 3T6 fibroblasts, were trypsinized, fixed in ethanol, and suspended in a solution containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/mL propidium iodide and treated for 30 min at 37° with 10 μg of RNase A. The stained cells were analyzed by a FACScan flow cytometer (Becton Dickin-

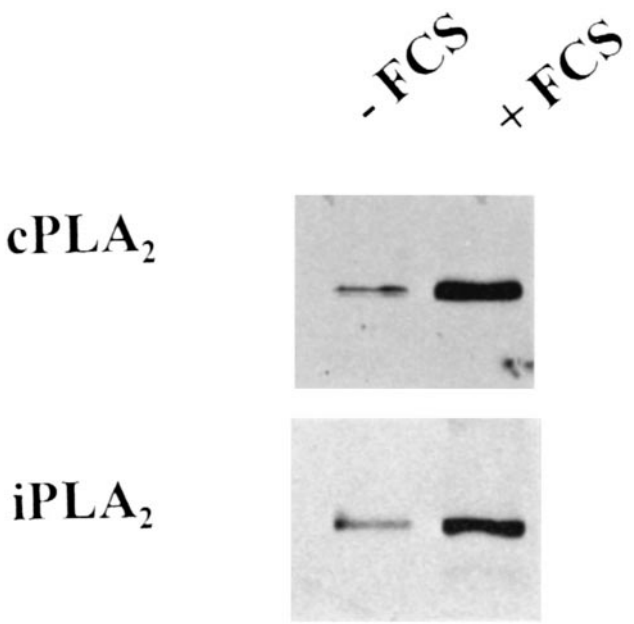

Fig. 1. Western blot analysis of $cPLA_2$ and $iPLA_2$ in cultured murine 3T6 fibroblast in absence (24 hr) or presence of FCS (10%). Western blot shown is representative of three experiments with similar results.

son), and the distribution of cells at each stage of the cycle was determined.

2.9. Statistical analysis

Results are expressed as means ± SEM. Differences between control cultures and treated cultures were tested by using either Student's *t*-test or one-way analysis of variance followed by the least significant difference test as appropriate.

3. Results

In order to determine the presence of $cPLA_2$ and $iPLA_2$ in 3T6 fibroblasts, we performed a Western-blotting analysis using specific antibodies. As shown in Fig. 1, antibodies recognized the presence of both enzymes.

Exposure to serum produced activation of PLA_2 that was manifested by [^{3}H]AA release (Fig. 2). $AACOCF_3$, a trimethylketone analogue of AA recently shown to strongly inhibit 85 kDa PLA_2 but not the 14-kDa form [16], significantly inhibited this [^{3}H]AA mobilization in 3T6 fibroblast cultures ($IC_{50} \sim 1$ μM) (Fig. 2). Recently, a selective $iPLA_2$ inhibitor BEL has been developed [17]. This drug also significantly reduced [^{3}H]AA release induced by serum ($IC_{50} \sim 2.5$ μM) (Fig. 2). Serum also increased the formation of PGE_2 by 3T6 fibroblasts. As indicated both the $cPLA_2$ inhibitor, $AACOCF_3$ and the $iPLA_2$ inhibitor, BEL produced significant decreases in PGE_2 in serum-supplemented media (Fig. 3), suggesting that both enzymes,

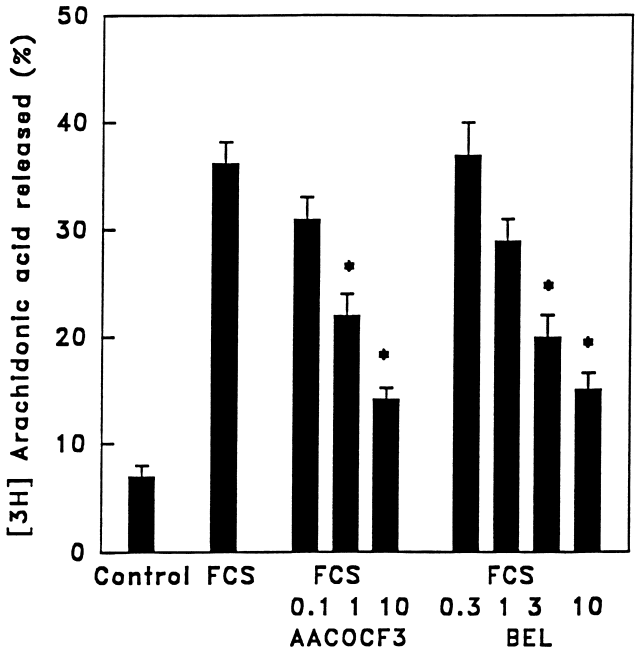

Fig. 2. Effect of $AACOCF_3$ (0.1–10 μM) and BEL (0.3–10 μM) on [^{3}H]AA release induced by FCS. Fibroblasts prelabeled with [^{3}H]AA were incubated with FCS (10%) in absence or presence of PLA_2 inhibitors for 2 hr. Data are the mean ± SEM (N = 5–6). $^*P < 0.05$ vs non-treated cells.

$cPLA_2$ and $iPLA_2$, contribute to AA release and the subsequent prostaglandin formation induced by FCS. However, these effects were significantly attenuated by the exogenous addition of AA (20 μM) to the media (Table 1).

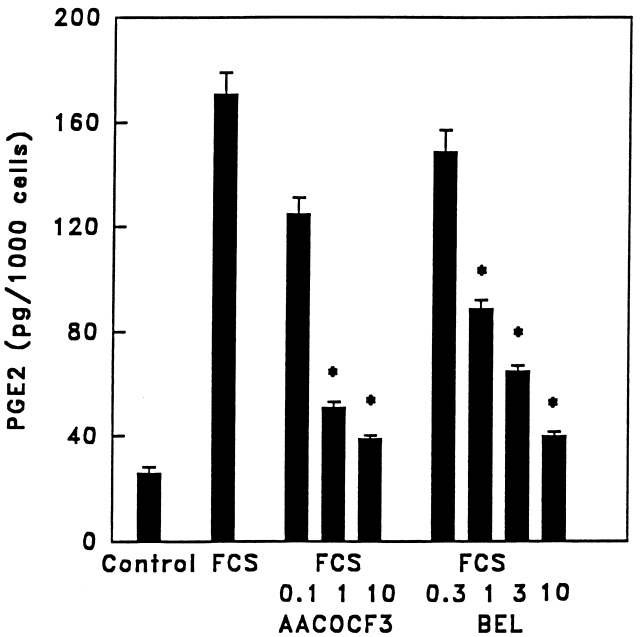

Fig. 3. Effect of $AACOCF_3$ (0.1–10 μM) and BEL (0.3–10 μM) on PGE_2 production stimulated by FCS. Fibroblasts were incubated with FCS (10%) in absence or presence of PLA_2 inhibitors for 2 hr. Data are the mean ± SEM (N = 4–5). $^*P < 0.05$ vs non-treated cells.

Table 1
Effect of $AACOCF_3$ and BEL on [^{3}H]AA release, PGE_2 production, and 3T6 growth induced by FCS

	% [^{3}H]AA released	PGE_2	3T6 growth ($\times 10^3$)
Control	8.2 ± 1.3	26 ± 3	
FCS	35.8 ± 2.1	162 ± 11	13.1 ± 1.2
FCS + AA		211 ± 9*	14.2 ± 1.1
FCS + $AACOCF_3$	15.1 ± 1.1*	38 ± 3*	3.9 ± 0.3*
FCS + $AACOCF_3$ + AA		112 ± 6**	7.6 ± 0.4**
FCS + BEL	19.7 ± 2.3*	62 ± 5*	7.8 ± 0.4*
FCS + BEL + AA		138 ± 9**	9.7 ± 0.3*

3T6 fibroblasts were maintained without FCS overnight. Then, we added FCS (20%) for 2 h. $AACOCF_3$ (10 μM) and BEL (3 μM) were added to the medium 30 min before FCS, and [^{3}H]AA released and PGE_2 (pg/1000 cells) were measured. In cell growth experiments, 3000 cells/well were incubated with FCS (10%) in presence or absence of $AACOCF_3$ (10 μM), BEL (3 μM) and AA (20 μM) for 3 days. All data are the mean ± SEM from two experiments performed in triplicate.

* $P < 0.05$ vs non-treated with PLA_2 inhibitors.

** $P < 0.05$ vs treatment without AA.

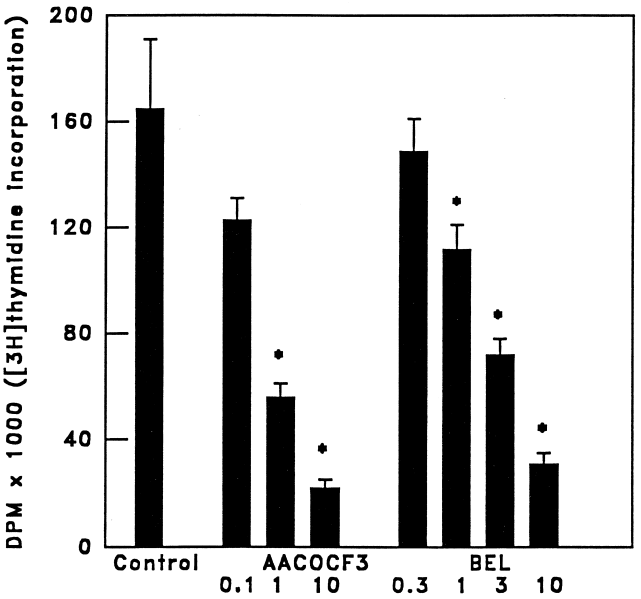

Fig. 5. [^{3}H]Thymidine incorporation induced by FCS was inhibited by $AACOCF_3$ and BEL. Data are the mean ± SEM (N = 3–4). *$P < 0.05$ vs non-treated cells. Dpm, disintegrations per minute.

3T6 fibroblast growth induced by FCS was also affected by $AACOCF_3$ in a dose-dependent manner. Recall that cells were seeded at a density of 3000 cells/well on day 0 and that by day 3, the cell number had increased 4.7-fold to 13980 ± 660 (Fig. 4). Our results show a dose-dependent inhibition of fibroblast growth induced by $AACOCF_3$ treatment (IC_{50} ~ 1 μM), and that 10 μM $AACOCF_3$ completely inhibited 3T6 fibroblast proliferation. BEL also significantly reduced 3T6 fibroblast growth in a dose-dependent manner (IC_{50} ~ 3 μM), and blocked 3T6 growth at 10 μM (Fig. 4).

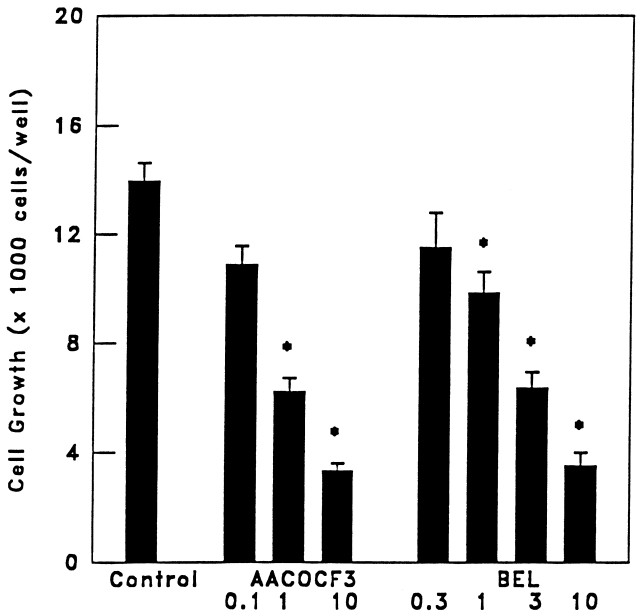

Fig. 4. High-molecular-weight PLA_2 inhibitors reduce 3T6 fibroblast proliferation induced by FCS. Cells were incubated with FCS (10%) in presence or absence of $AACOCF_3$ (0.1–10 μM) or BEL (0.3–10 μM) for 3 days. Data are the mean ± SEM (N = 5–6). *$P < 0.05$ vs non-treated cells.

Serum produced a significant increase in the rate of [^{3}H]thymidine incorporation in 3T6 fibroblasts. $AACOCF_3$ and BEL inhibited DNA synthesis in a dose-dependent manner, measured as [^{3}H]thymidine incorporation (Fig. 5).

The trypan blue exclusion test indicated that the $AACOCF_3$- and BEL-mediated reduction of fibroblast growth was not due to cellular toxicity. Moreover, a morphologic examination demonstrated that the drug treatments produced no changes in cell structure (data not shown).

Interestingly, the effect of $AACOCF_3$ and BEL on 3T6 fibroblast growth was significantly attenuated by addition of AA to the media (Table 1). Moreover, 3T6 fibroblasts recovered their growth rate when $AACOCF_3$ was removed from the media (Fig. 6). Similar data were obtained with BEL, although the recovery of the growth rate appeared latter than when we removed $AACOCF_3$.

Fibroblasts were collected and stained with propidium iodide after 3 days of exposure to the inhibitors to determine the effect of treatments on the cell cycle. Fig. 7 provides data from a representative experiment that show that $AACOCF_3$ and BEL caused the impairment of the G_1 peak and the increase of cells in S phase. However, manoalide, a secreted PLA_2 inhibitor [18], and flosulide, a cyclooxygenase-2 inhibitor [19] did not modify 3T6 fibroblast cycle. These changes were not due to the induction of cell death and were fully reversible when we removed PLA_2 inhibitors (data not shown).

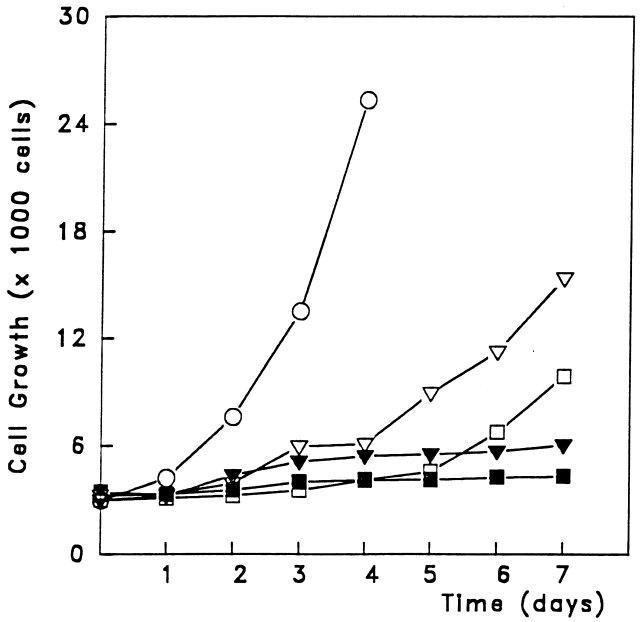

Fig. 6. The inhibition of $AACOCF_3$ or BEL on fibroblast growth were reversible. Cells were treated with $AACOCF_3$ (10 μM, ▼) or BEL (10 μM, ■) for 7 days or removed after 2 days (▽, □) in presence of 10% FCS. Control cells were cultured in presence of 10% FCS (○). Data are the mean of 3–4 results.

4. Discussion

AA release is an early event in the mitogenic process of a number of growth factors and the subsequent cell proliferation. This mobilization of AA from membrane phospholipids occurs mainly by two pathways: hydrolytic cleavage of the *sn*-2 position of the glycerophospholipid backbone catalyzed by PLA_2, or activation of phospholipase C, which cleaves the phosphate-ester bond, and subsequent hydrolysis of diacylglycerol produced by diacylglycerol lipase. In 3T6 fibroblasts, stimulated AA release through PLA_2 has been shown to be required for mitogenesis. Moreover, these previous studies demonstrated that nonselective PLA_2 inhibitors reduce 3T6 fibroblast proliferation [13,15].

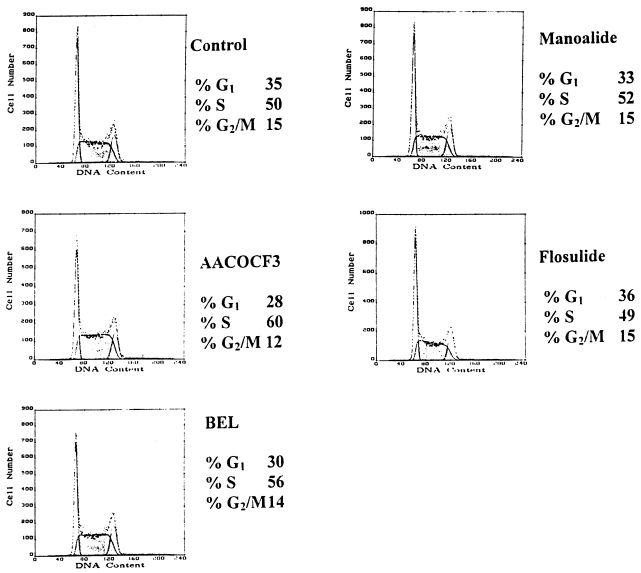

Fig. 7. Distribution of 3T6 fibroblast with respect to DNA content in cells cultured with FCS (10%) for 2 days or FCS in presence of $AACOCF_3$ (10 μM), BEL (10 μM), manoalide (0.5 μM) or flosulide (10 μM). This experiment is representative of three experiments with similar results.

Owing to the central role of $cPLA_2$ in AA signaling, the design of $cPLA_2$ inhibitors is an area of great interest. $AACOCF_3$ is a reversible $cPLA_2$ inhibitor that also inhibits cyclooxygenases [16]. Unfortunately, $AACOCF_3$ also potently inhibits $iPLA_2$ [20] because both $cPLA_2$ and $iPLA_2$ appear to use a central Ser for catalysis and, probably, similar catalytic mechanisms. There is one way to ascertain whether the inhibitory effects of $AACOCF_3$ result from $cPLA_2$ or $iPLA_2$. This involves the parallel use of BEL, a selective inhibitor of $iPLA_2$ [17]. Thus, if a given response is inhibited by $AACOCF_3$ and by BEL, this would indicate the involvement of $iPLA_2$ [21].

Our results suggest that BEL-sensitive $iPLA_2$ may be, at least partially, implicated in the AA release, PGE_2 generation and cell growth stimulated by FCS in 3T6 fibroblast cultures, in agreement with recent studies with $iPLA_2$ inhibitors including BEL that suggested the contribution of $iPLA_2$ to stimulus-induced AA liberation [22,23]. However, further studies will be necessary to understand the precise role of $cPLA_2$ and $iPLA_2$ on FCS-stimulated AA release and 3T6 fibroblast growth.

Interestingly, FACS-scan analysis suggests that the reduction in cell growth following $AACOCF_3$ and BEL treatments results from an impairment of the percentage of cells in G_1 and the enhancement of cells in S phase whereas manoalide and flosulide, treatments that partially reduced 3T6 growth [15], did not induce changes.

The mechanism by which high-molecular-weight PLA_2 influences 3T6 fibroblast proliferation remains to be determined. However, cyclooxygenase inhibitors such as ketoprofen or indomethacin had effect on 3T6 fibroblast growth [15,24], indicating that the high-molecular-weight PLA_2-dependent effect on proliferation may be mediated by AA and/or prostaglandins. Thus, when fibroblasts were treated with exogenous AA, the reduction in growth rate produced by $AACOCF_3$ or BEL was significantly attenuated. This action may be related to the capacity of AA to stimulate *c*-fos, *c*-jun, and/or mitogen-activated protein kinase [25, 26]. Additionally, recent reports indicate that the release of AA by PLA_2 is accompanied by the formation of lysolipids, and lysophosphatidic acid, lysophosphatidylinositol and lysophosphatidylcholine stimulate mitogen-activated protein kinases and proliferation [27,28]. Moreover, these lysolipids are precursors of glycerophosphoinositides, which show evidence of being accumulated in ras-transformed cells.

In summary, we have demonstrated that $AACOCF_3$ and BEL block 3T6 fibroblast proliferation and DNA synthesis at the S/G_2 boundary, thus prolonging the S phase. These

data suggest a role of $iPLA_2$ in the control of 3T6 fibroblast growth.

Acknowledgments

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