Role of EP$_1$ and EP$_4$ PGE$_2$ subtype receptors in serum-induced 3T6 fibroblast cycle progression and proliferation

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Received 9 March 2001; accepted in final form 15 October 2001

Sanchez, Teresa, and Juan Jose Moreno. Role of EP$_1$ and EP$_4$ PGE$_2$ subtype receptors in serum-induced 3T6 fibroblast cycle progression and proliferation. Am J Physiol Cell Physiol 282: C280–C288, 2002. First published October 3, 2001; 10.1152/ajpcell.00128.2001.—Recent studies have suggested that prostaglandin E$_2$ (PGE$_2$) subtype receptors (EP) are involved in cellular proliferation and tumor development. We studied the role of EP$_1$ and EP$_4$ PGE$_2$ subtype receptor antagonists AH-6809 and AH-23848B, respectively, in serum-induced 3T6 fibroblast proliferation. This was significantly reduced in a dose-dependent manner (IC$_{50}$ ~100 and ~30 μM, respectively) to an almost complete inhibition, without any cytotoxic effect. However, the effect of each antagonist on 3T6 cell cycle progression clearly differed. Whereas the EP$_1$ antagonist increased the G$_0$/G$_1$ population, the EP$_4$ antagonist brought about an accumulation of cells in early S phase. These effects were associated with a decrease in cyclin D and E levels in AH-6809-treated 3T6 cells and lower cyclin A levels in AH-23848B-treated fibroblasts with respect to control cells. The G$_0$/G$_1$ accumulation caused by AH-6809 seems to be intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) dependent, because a 6-h 1 μM thapsigargin treatment allowed G$_0$/G$_1$-arrested cells to enter S phase. Similarly, treatment with 20 μM forskolin for 6 h allowed S-phase and G$_2$/M progression of AH-23848B-treated cells. This study shows that the inhibitory effect of the EP$_1$ and EP$_4$ antagonists on serum-induced 3T6 fibroblast growth is due to their effect at various levels of the cell cycle machinery, suggesting that PGE$_2$ interaction with its different subtype receptors regulates progression through the cell cycle by modulating cAMP and [Ca$^{2+}$]$_i$.

Arachidonic acid; cyclooxygenase; prostaglandin G/H synthase; phospholipase A$_2$

Arachidonic acid (AA) is predominantly esterified in the phospholipids of membranes, and it is mobilized by phospholipases, especially phospholipases A$_2$ (PLA$_2$s). Once released, free intracellular AA can be oxidized via three major metabolic pathways: the prostaglandin G/H synthase (PGHS), the lipoygenase, and the cytochrome P-450 monoxygenase pathways. Thus AA is the precursor of a large number of biologically active molecules collectively named eicosanoids (34). PGHS, also known as cyclooxygenase, catalyzes the conversion of AA to prostaglandin H$_2$ (PGH$_2$), the immediate precursor of prostanoids. Two isoforms of PGHS have been identified and cloned in eukaryotic cells (30). PGHS-1 is encoded by a housekeeping gene and is thought to be involved in the maintenance of physiological functions, whereas the expression of PGHS-2 is induced by various stimuli, including mitogens, cytokines, and bacterial lipopolysaccharides (17). The overexpression of PGHS-2 has been associated with a variety of proliferative diseases, such as colorectal, gastric, and breast cancer (12).

Prostanoids have numerous physiological effects (48), including cell proliferation and differentiation. PGEs induce DNA synthesis in Swiss 3T3 fibroblasts (41). Furthermore, enhanced synthesis of PGE$_2$ was observed in human breast cancer, in experimental murine mammary tumor tissues (45, 52), and in human colon cancer tissue, compared with surrounding normal mucosa (39). PGE$_2$ also may inhibit programmed cell death and enhance the tumorigenic potential of colonic epithelial cells (47). We also have suggested elsewhere that PGE$_2$, the major cyclooxygenase pathway metabolite produced by fibroblasts (22), controls 3T6 fibroblast growth and that PGHS-2 is the isoform responsible for serum-induced PGE$_2$ synthesis in these cells (29).

The four subtypes of PGE receptors (EP$_1$, EP$_2$, EP$_3$, and EP$_4$) from various species, previously defined pharmacologically, have recently been cloned (see Ref. 33 for review). They are encoded by a distinct gene and differ in their amino acid identities, pharmacological characteristics, and signal transduction properties. The unique coupling of EP subtypes to a given signal transduction pathway provides the molecular basis for the diverse and complex physiological roles of PGE$_2$. Thus binding of PGE$_2$ to its receptor alters the levels of second messengers such as intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) (61) and cAMP (41).

Several studies have suggested the role of PGE$_2$ receptors in cellular proliferation and tumor development. The EP$_3$ subtype PGE$_2$ receptor is responsible for the proliferative effect of PGs on rat hepatocytes (16). Experiments on EP$_1$-deficient mice show that this subtype receptor is required for the development of preneoplastic lesions induced in colon epithelium by...
the carcinogen azoxymethane (56). The same authors have proposed a chemopreventive effect of the EP1-selective antagonist ONO-8713 on colon cancer (55).

We aimed to clarify the role of PGE2 interaction with EP1 and EP4 subtype receptors in 3T6 fibroblast growth. Both EP1 and EP4 are expressed in murine fibroblasts (57). To that end, we used specific antagonists, AH-6809 as an EP1 antagonist (8) and AH-23848B to block PGE2 interaction with its EP4 receptor (5). The EP1 receptor mediates PGE2-induced elevation of free Ca2+, whereas the EP4 receptor is coupled to Gs and mediates increases in cAMP concentration by activation of adenylyl cyclase (9). Both Ca2+ and cAMP play a pivotal role as mediators of cell cycle progression. The Ca2+ signaling system is believed to activate the immediate-early genes responsible for inducing resting cells to reenter the cell cycle (2). On the other hand, the contribution of the cAMP signaling pathway to cell growth seems to be dependent on both cell type and cell cycle status. Progression through the mammalian cell cycle is controlled at various levels by cyclins, cyclin-dependent kinases (Cdks), and cyclin-dependent kinase inhibitors (CKIs) (38). Cdks form a binary system composed of an inactive catalytic subunit (Cdk), which is activated by its binding to the regulatory subunit (cyclin). Thus the first level of regulation of its activity is cyclin availability, which is controlled by transcriptional regulation, mRNA stability, and ubiquitin-dependent degradation. Cdk activity also depends on positive and negative regulatory phosphorylations and Cdk inhibitors: the Cip/Kip family, which downregulates the activity of all cyclin-Cdk complexes, and the INK4 family, which includes specific inhibitors of Cdk4 and 6. Cyclin D family members control cell cycle exit (62) and are essential components of an early G1 checkpoint (37). Their expression depends on continuous mitogenic stimulation. Progression through G1 of the cell cycle is governed by cyclin D-Cdk4/6 complexes in early G1 phase and cyclin E-Cdk2 complexes in late G1 (for review see Ref. 15). Cyclin E mRNA and protein begin to accumulate in late G1, peak at the G1/S transition, and are downregulated during S phase (23). Cdk2-cyclin E activity is required for progression to S phase (36). At the G1-S phase transition, the tumor suppressor pRB, product of the retinoblastoma gene, is inactivated by hyperphosphorylation by the complex cyclin D-Cdk4/6, which allows the transcription factor family E2F to activate E2 site-containing gene promoters (see Ref. 13 for review), leading to S-phase gene transcription. Cyclin A is an S-phase cyclin, which, with its partner Cdk2, associates with the transcription factor E2F1/DP1. This association is crucial for the transit of cells through the S-phase checkpoint (25).

Our results suggest that interaction of PGE2 with both EP1 and EP4 subtype receptors accounts for its role in 3T6 fibroblast growth regulation. The growth-inhibitory effect of EP1 and EP4 PGE2 receptor antagonists on serum-stimulated cellular proliferation seems to involve Ca2+ and cAMP, respectively, acting at distinct levels of the cell cycle machinery. Thus, when PGE2 interaction with EP1 was blocked, cyclin D and E levels decreased and 3T6 cells were arrested in G0/G1 phase. In contrast, blocking PGE2 interaction with EP4 receptor reduced cyclin A levels and induced an early S-phase arrest in 3T6 cells.

**MATERIAL AND METHODS**

**Reagents.** RPMI 1640, fetal calf serum (FCS), penicillin G, streptomycin, and trypsin/EDTA were from BioWhittaker Europe (Verviers, Belgium). AH-6809 (6-isopropoxy-9-oxoanthene-2-carboxylic acid) and AH-23848B ([1α,2β,5α]-(±)-7-[(1,1-biphenyl-4-yl)metoxy]-2-(4-morphonyl)-3-methoxocyclopentil-4-heptenoic acid) were kindly provided by Glaxo Wellcome (Stevenage, UK). Aprotinin, leupeptin, diethylthiocarbamic acid, phenylmethylsulfonyl fluoride (PMSF), NaF, Na3VO4, Igepal CA-630, dithiothreitol (DTT), forskolin, propidium iodide (PI), ethidium bromide, acridine orange, and DNase-free RNase A were acquired from Sigma Chemical (St. Louis, MO). Rabbit polyclonal antibodies against cyclins D, E, and A were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell culture.** Murine 3T6 fibroblasts (ATCC CCL96) were grown, as described elsewhere (29), in RPMI 1640 containing 10% FCS (10% FCS-RPMI), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 95% air-5% CO2 at 37°C. Cells were harvested with trypsin/EDTA and passed to tissue culture 100- or 60-mm dishes (Costar, Cambridge, MA) for experimental purposes.

**Cell growth.** The effect of prostanoid receptor antagonists was assessed on 3T6 fibroblasts plated at 104 cells/well in 12-well plates (Costar) and cultured for 24 h in 10% FCS-RPMI. Cells were incubated for 2 days in 10% FCS medium in the presence of the different treatments. At the end of the experiments, cells were washed, trypsinized, and counted by using ethidium bromide/acridine orange stain to assess viability. To study cell recovery, after a 24-h treatment with AH-6809 or AH-23848B, 3T6 fibroblast cultures were washed with phosphate-buffered saline solution (PBS) and fresh 10% FCS medium was added. Growth recovery was assessed after 1, 2, or 3 days by counting trypsinized cells, as mentioned above.

**Fluorescence-activated cell sorting analysis/flow cytometry cell cycle analysis.** Cells were seeded, and 24 h later they were treated with PBS and serum starved. After 24-h serum starvation, the percentage of cells in G0/G1 was ~50%. Cells were then cultured in 10% FCS-RPMI containing the treatments. After 30 h, cells were trypsinized, fixed with 70% ethanol, and stored at 4°C for at least 2 h. Thapsigargin and forskolin were added to the AH-6809 or AH-23848B-containing culture medium after a 30-h incubation, and 6 h later, cells were harvested and fixed. They were then stained for 1 h at room temperature with a 20 μg/ml PI solution in PBS containing 0.1% Triton X-100 (Sigma) and 0.2 mg/ml DNase-free RNase A. Cells were analyzed on an Epics XL flow cytometer (Coulter, Hialeah, FL). The instrument was set up with the standard configuration: excitation of the sample was performed with a standard 488-nm air-cooled argon-ion laser at 15 mV. Forward scatter, side scatter, and red (620 nm) fluorescence for PI were acquired. Optical alignment was based on optimized signal from 10-nm fluorescent beads (Immunochek, Epics Division). Time was used as a control of the stability of the instrument. Red fluorescence was projected on a 1024 monoparametrical histogram. Aggregates were excluded by gating single cells by their area vs. peak fluorescence signal. DNA was analyzed (Ploidy analysis) on
onists in serum-induced 3T6 fibroblast proliferation. To determine the role of PGE_2 on 3T6 fibroblast proliferation through the interaction with its G protein-coupled receptors, EP_1 and EP_4, the cells were cultured in 10% FCS-RPMI in the presence of the antagonists AH-6809 and AH-23848B, respectively (5, 8), for 48 h.

Cell growth was significantly reduced by both compounds in a dose-dependent manner (Fig. 1). The IC_{50} of AH-6809 (~100 μM) was higher than that of AH-23848B (~30 μM). Interestingly, both antagonists almost completely inhibited cell growth at the highest doses used. The above-mentioned treatments did not cause cytotoxicity at the concentrations and for the periods of time assayed, as was assessed by observing morphological appearance of the cells by light microscopy and ethidium bromide/acridine orange staining. Moreover, once the treatments were removed from the culture medium, cells recovered the growth (Figs. 2 and 3), although AH-23848B-treated cells did so at a slower rate. Thus the effect of these antagonists is due to growth inhibition and not to the cytotoxic effect of the treatments.

**Effect of EP_1 and EP_4 subtype PGE_2 receptor antagonists on cell cycle distribution of 3T6 fibroblasts.** Next, we determined the effect of EP_1 and EP_4 antagonists on cell cycle progression of 3T6 fibroblasts. 3T6 fibroblast cultures that had been G_0/G_1 synchronized by overnight serum starvation were incubated with 10% FCS medium containing AH-6809 or AH-23848B for 30 h, a period of time that corresponds to approximately two cycles in 3T6 cells. Cell cycle distribution was analyzed by DNA staining with PI, followed by fluorescence-activated cell sorting (FACS) analysis.

**Statistical analysis.** Results are expressed as means ± SE. Differences between nontreated and treated cells were tested by using Student's t-test followed by the least significant difference test as appropriate.

**RESULTS**

**Effect of EP_1 and EP_4 subtype PGE_2 receptor antagonists in serum-induced 3T6 fibroblast proliferation.** To determine the role of PGE_2 on 3T6 fibroblast proliferation through the interaction with its G protein-coupled receptors, EP_1 and EP_4, the cells were cultured in 10% FCS-RPMI in the presence of the antagonists AH-6809 and AH-23848B, respectively (5, 8), for 48 h.
population increased from 53% in control cells to 86% in 100 μM AH-23848B-treated cells, and there was a decrease in G2/M (to 32% in control cells to 10% in 100 μM AH-23848B-treated cells) (Fig. 4). These results indicate that both antagonists inhibit serum-induced 3T6 fibroblast growth at distinct levels of the cell cycle, suggesting that PGE2 specifically regulates cellular proliferation in these cells by interaction with its different subtype receptors. Interestingly, when 24-h serum-starved synchronized 3T6 fibroblasts (80 ± 5% in G0/G1, 15 ± 4% in S, and 5 ± 4% in G2/M) were treated with 10% FCS media in the presence of both antagonists (30 μM AH-23848B and 100 μM AH-6809), cells went through G1 and finally accumulated in S phase (78 ± 5%), whereas a very low percentage remained in G2/M (1 ± 0.5%). This suggests that the S-phase arrest induced by the EP4 antagonist is stronger and more limiting than the G0/G1 arrest induced by the EP1 antagonist, because AH-6809 plus AH-23848B treatment barely allowed G0/G1-synchronized cells to complete S phase, whereas they seemed to progress through G0/G1 and enter in S phase, although more slowly than control cells.

Finally, note that none of the treatments we applied caused a population of cells with DNA content less than 2n, as would be expected if some of them induced cell apoptosis.

Effect of AH-6809 and AH-23848B on cyclin levels. Progression of the mammalian cell cycle is governed by cyclins, Cdks, and CKIs (38). Cdks form a binary system composed of the inactive catalytic subunit Cdk, which is activated by binding to cyclin. We aimed to determine whether the impaired progression through the cycle of EP1 or EP4 antagonist-treated cells was linked to changes in G1 and S phase cyclin levels, which control transition between the phases of the cell cycle in association with Cdks.

The levels of cyclin D, E, and A protein were low in quiescent 3T6 cells. Exposure of these cells to 10% FCS-containing media caused a time-dependent increase of cyclin D (maximum value within 6–18 h), cyclin E (maximum value within 4–10 h), and, thereafter, cyclin A protein (maximum value at 18 h) (Fig. 5). We next determined the effect of 150 μM AH-6809 and 100 μM AH-23848B on serum-induced cyclin D, E,
and A expression in G0/G1-synchronized 3T6 fibroblast cultures after 6, 6, and 18 h, respectively, of serum-containing medium addition, time points at which a significant and consistent expression of these cyclins was observed. As shown in Fig. 6, AH-6809 significantly reduced cyclin D and E levels, whereas AH-23848B had no effect on the levels of these cyclins. In contrast, this compound significantly decreased cyclin A expression without affecting cyclin D and E levels (Fig. 6).

Our results indicate that AH-6809 and AH-23848B interfere with cell cycle progression, resulting in growth arrest in defined phases and impaired cyclin levels. Both EP1 and EP4 thus may be involved in the regulation of 3T6 fibroblast growth by PGE2, but at different levels of the cell cycle machinery.

\[ \text{Ca}^{2+} \text{ and cAMP are involved in PGE}_2 \text{ regulation of 3T6 fibroblast growth.} \]

Because the EP1 receptor mediates PGE2-induced elevation of free [\( \text{Ca}^{2+} \)] \( i \), and the EP4 receptor is coupled to \( G_s \) and increases cAMP concentration by activation of adenyl cyclase (9), the regulation of serum-stimulated 3T6 fibroblast proliferation by PGE2 may involve \( \text{Ca}^{2+},\text{- and cAMP-dependent mechanisms.} \)

Some experiments were performed to test this hypothesis. We used thapsigargin and forskolin, an adenylyl cyclase activator (46), to increase [\( \text{Ca}^{2+} \)] \( i \) and cAMP concentrations, respectively. Thapsigargin activates the release of \( \text{Ca}^{2+} \) from intracellular stores by inhibiting the ATPase-driven uptake of \( \text{Ca}^{2+} \) in the endoplasmic reticulum. Thus it induces the entry of this cation from extracellular space (51). Thapsigargin or forskolin was added 6 h before the cells were harvested and treated with EP1 or EP4 antagonist for 30 h, respectively. Thapsigargin (1 \( \mu \)M) allowed 3T6 cells to overcome the cell cycle arrest in G0/G1 induced by 150 \( \mu \)M AH-6809 treatment (47 ± 4.5% in G0/G1 compared with 61 ± 11% without thapsigargin treatment) (Fig. 7). Similarly, forskolin (20 \( \mu \)M) treatment allowed 100 \( \mu \)M AH-23848B-treated 3T6 fibroblasts cultures arrested in early S phase to progress through the cell cycle (49 ± 0.5% of cells in S phase compared with 86 ± 5% without forskolin treatment).

The effect of thapsigargin and forskolin on cell cycle progression was correlated with a recovery of 3T6 cell growth (Table 1). These results support the hypothesis that PGE2 governs progression through distinct stages of the cell cycle and proliferation of serum-stimulated 3T6 fibroblasts by regulating [\( \text{Ca}^{2+} \)] \( i \) and cAMP levels.

**DISCUSSION**

The PGHS pathway of the AA cascade has been suggested to play a pivotal role in cell proliferation and in the development of numerous human tumors, such as colon, breast, and lung carcinoma (12).

Murine fibroblasts have served as a model system for the study of the regulation of mitogenesis. We have reported elsewhere that a \( \text{Ca}^{2+},\text{-independent cytosolic PLA}_{2} \) is involved in serum-induced 3T6 fibroblast proliferation (43). Furthermore, in these cells, PGE2, which is synthesized mainly by the inducible form of PGHS, plays a key role in the control of FCS-induced growth (29). On the other hand, PGs have been shown to stimulate 3T3 fibroblast DNA synthesis via PGF2\( \alpha \) receptor (FP), EP1 subtype receptor, and PGI2 (IP) through \( \text{Ca}^{2+},\text{- and cAMP-dependent pathways (57).} \)

We aimed to elucidate the effect of PGE2 interaction with its EP1 and EP4 subtype receptors on the control of serum-induced 3T6 fibroblast proliferation and on progression through the cell cycle. Both EP1 and EP4 PGE2 subtype receptors have been found in murine fibroblasts (57). Mouse EP1 and EP4 subtype receptors have been cloned by Ichikawa and colleagues (18, 54). Interestingly, EP1 was first cloned as an EP2 receptor, but it was later shown to be sensitive to an EP4-specific ligand, AH-23848B (35). Here, we chose two specific antagonists, AH-6809, an EP1 subtype antagonist (8), and AH-23848B, to block PGE2 interaction with EP4 subtype receptors (5). Both of them reduced serum-induced 3T6 fibroblast growth in a dose-dependent manner, and almost blocked cellular proliferation, without showing cytotoxicity at the concentrations and for the incubation times used. In fact, when treatments were withdrawn, cells recovered the growth. However, higher doses of the EP1 antagonist were required to block cellular proliferation. This may be due to the lower affinity of AH-6809 for the mouse EP1 receptor, compared with its affinity for the human receptor (54). These results agree with recent studies suggesting that PGE2 affects cellular proliferation and cancer development by interaction with specific subtype receptors.

![Figure 6](http://ajpcell.physiology.org/)
PGE2 induces the expression of early response genes, such as c-fos, c-jun, jun B, and egr-1, leading to enhanced proliferation and/or differentiation of osteogenic precursors (60). This effect is abolished by AH-23848B in a dose-dependent manner, suggesting that it is cAMP dependent (59).

The role of PGE2 interaction with EP1 subtype receptor in colon carcinogenesis has been reported with the use of EP1 knockout mice (56) and an EP1-specific antagonist (55). We have shown elsewhere that S-(+/-)-ketoprofen, a dual inhibitor of both cyclooxygenase isoforms, significantly reduces the serum-induced synthesis of PGE2 in 3T6 fibroblasts and cell growth in a dose-dependent manner (42). However, this inhibition reaches a plateau at 50–60% (maximum inhibition). Here, the EP1 and EP4 antagonist treatment almost blocked 3T6 fibroblast growth.

When the synthesis of prostaglandins is abolished by a cyclooxygenase inhibitor, the AA released after mitogenic stimuli by PLA2s can be metabolized by lipoxygenases and cytochrome P-450 enzymes. Some lipoxygenase and cytochrome P-450 monoxygenase AA metabolites have been shown to activate protein kinase C and members of the mitogen-activated protein kinase family (4, 32, 49). The mitogenic effect of other AA metabolites, which can be formed when the synthesis of PGs is blocked, may explain the distinct effect of PGHS inhibitors and PGE2 antagonists on 3T6 cell proliferation. Further studies should be performed to clarify the role of lipoxygenase and cytochrome P-450 pathways on 3T6 fibroblast growth.

In the present study, we also provide evidence that the EP1 and EP4 antagonists differentially affect 3T6 fibroblast cycle progression, suggesting that PGE2 reg-

### Table 1. Effect of thapsigargin and forskolin in cell growth arrest induced by AH-6809 and AH-23848B

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell Number, ×10⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>451 ± 13</td>
</tr>
<tr>
<td>Control + thapsigargin (10 h)</td>
<td>422 ± 11</td>
</tr>
<tr>
<td>AH-6809</td>
<td>121 ± 6*</td>
</tr>
<tr>
<td>AH-6809 + thapsigargin (6 h)</td>
<td>146 ± 6†</td>
</tr>
<tr>
<td>AH-6809 + thapsigargin (10 h)</td>
<td>161 ± 8†</td>
</tr>
<tr>
<td>Control + forskolin (10 h)</td>
<td>387 ± 15</td>
</tr>
<tr>
<td>AH-23848B</td>
<td>127 ± 6*</td>
</tr>
<tr>
<td>AH-23848B + forskolin (6 h)</td>
<td>136 ± 5</td>
</tr>
<tr>
<td>AH-23848B + forskolin (10 h)</td>
<td>152 ± 5†</td>
</tr>
</tbody>
</table>

3T6 fibroblast cultures synchronized by 24-h serum starvation were incubated with 10% FCS-RPMI (control) or 10% FCS-RPMI containing 150 μM AH-6809 or 100 μM AH-23848B for 30 h. Thapsigargin (1 μM) or forskolin (20 μM) was added to dishes containing 150 μM AH-6809 (AH-6809 + Thaps) or 100 μM AH-23848B (AH-23848B + Forsk), respectively, and cells were incubated for a further 6 h. After treatments, cells were trypsinized, counted, and results are means ± SE from 3 experiments performed in triplicate. *P < 0.05 vs. nontreated cells. †P < 0.05 vs. antagonist-treated cells.
ulators cellular proliferation at various levels of the cell cycle machinery through its interaction with specific subtype receptors, thus modifying different second messenger systems. The EP<sub>4</sub> antagonist caused an early S-phase arrest of 3T6 fibroblasts, whereas the EP<sub>1</sub> antagonist treatment of these cells was associated with a significant increase in G<sub>0</sub>/G<sub>1</sub> population with respect to control cells. However, we cannot rule out the presence of EP<sub>2</sub> or EP<sub>3</sub> receptors in 3T6 fibroblasts and their contribution to the control of their growth, because it has been described in other cell types (18, 24). The hypothesis that PGE<sub>2</sub> regulates the cell cycle at different levels by interacting with specific subtype receptors also was supported by the data on the effect of AH-6809 and AH-23848B on cyclin levels. Thus the increase in G<sub>0</sub>/G<sub>1</sub> population of 3T<sub>6</sub> fibroblast cultures induced by the EP<sub>1</sub> antagonist was linked to a decrease in cyclin D and E protein levels, and the early S-phase arrest induced by the EP<sub>4</sub> antagonist was linked to a significant decrease in cyclin A levels. Progression of the cell cycle through G<sub>1</sub> is governed by cyclin D-Cdk4/6 complexes in early G<sub>1</sub> and by cyclin E-Cdk2 complexes in late G<sub>1</sub> (for review, see Ref. 15). Thus the lower levels of cyclins D and E may explain the G<sub>0</sub>/G<sub>1</sub> arrest induced by the EP<sub>1</sub> antagonist in our experimental conditions. On the other hand, cyclin A, an S-phase cyclin, is also essential for progression of the mammalian cell cycle. First, the complex cyclin A-Cdk2 binds and phosphorylates the replication protein A, which is critical for DNA replication (14). Second, cyclin A2 forms a complex with the E2F family of transcription factors during S phase and regulates its transcriptional activity (58). Phosphorylation of E2F-bound DP1 by cyclin A-Cdk2 leads to suppression of E2F-1 DNA-binding activity, resulting in the transit of cells through the S-phase checkpoint (25). Dysfunctional cyclin A-Cdk2-E2F-1 interaction during S phase leads to arrest in early S phase (25, 50). These findings agree with the early S-phase arrest found in 3T6 fibroblasts after AH-23848B treatment that may result from the decrease in cyclin A levels. Previous studies have shown that PGF<sub>2a</sub> induced cyclin D1 expression and DNA synthesis in 3T3 cells (44), but to our knowledge this is the first report on PGE<sub>2</sub> controlling progression through different phases of the cell cycle by interacting with two different subtype receptors.

The cellular response to PGE<sub>2</sub> is controlled by changes in second messenger levels, induced by its interaction with its receptors. The EP<sub>1</sub> receptor mediates PGE<sub>2</sub>-induced elevation of free Ca<sup>2+</sup> with a barely detectable phosphatidylinositol response (54), suggesting that it may regulate Ca<sup>2+</sup> channels via an unidentified G protein. In turn, the EP<sub>4</sub> receptor is coupled to G<sub>s</sub> and increases cAMP concentration by activation of adenyl cyclase (9). However, little is known about the role of PGE<sub>2</sub> in regulating progression through the cell cycle by its interaction with its distinct subtype receptors. Our studies suggest that PGE<sub>2</sub> controls cellular proliferation via [Ca<sup>2+</sup>], and cAMP. Both messengers seem to be crucial at different stages of the cell cycle. Thus 6-h thapsigargin treatment partially counteracted the G<sub>0</sub>/G<sub>1</sub> arrest of 3T6 fibroblasts induced by AH-6809, suggesting a role for Ca<sup>2+</sup> levels in the transition from G<sub>0</sub> to G<sub>1</sub> and S phases and, consequently, the growth arrest induced by the EP<sub>1</sub> antagonist. These results agree with the findings of Morris et al. (31), who found that Ca<sup>2+</sup> and calmodulin were necessary for G<sub>1</sub> progression in fibroblasts. Calmodulin kinase II inhibition reduced cyclin D1 levels, causing G<sub>1</sub> arrest in NIH/3T3 cells. The lower cyclin E levels that we also have found may be a consequence of the decrease in cyclin D levels, because cyclin E transcription is activated when pRb is hyperphosphorylated by the complexes cyclin-D-Cdk4/6 and no longer inhibits E2F/DP.

cAMP may be involved in S-phase progression of 3T6 fibroblasts by regulating cyclin A levels. The intracellular levels of cyclin A protein are regulated mainly at the levels of transcription and/or mRNA stability (28). The human and mouse cyclin A genes have recently been cloned and their promoters analyzed (19). The cyclin A ATF site is bound by activating transcription factor-1 (ATF-1) and the cAMP-response element binding protein (CREB), which function as positive regulators of the cyclin A promoter. This transcription factor binds to its corresponding site and mediates the stimulatory effect of a cAMP analog on cyclin A gene transcription in human fibroblasts (10). The activity of ATF-1 and CREB also is regulated by their phosphorylation status (21). Indeed, transforming growth factor-β1, which inhibits proliferation of most normal cell types (1), induces growth arrest in mink lung epithelial cells by decreasing the phosphorylation of CREB and ATF-1 and thus downregulating cyclin A promoter activity. However, the role of cAMP in cell proliferation seems to be controversial. In certain cell lines it behaves as a mitogenic stimulus (40), whereas in other cells it induces cell cycle arrest (53). Our results agree with those of Lee et al. (27) and Desdouets et al. (11), who suggested that cAMP regulates cyclin A levels. Therefore, the contribution of the cAMP signaling pathway to cell growth may thus depend on both cell type and cell cycle status.

In conclusion, we provide evidence indicating that the effect of PGE<sub>2</sub> interaction with EP<sub>1</sub> and EP<sub>4</sub> subtype receptors on 3T6 fibroblast progression is associated with changes in specific cyclin levels, which control cell cycle progression. EP<sub>1</sub> and EP<sub>4</sub> antagonists decreased cyclin D, E, and A protein levels, the respective first levels of regulation of Cdk-4, Ckd-6, and Cdk-2 activity. These changes may explain the cell cycle arrest induced by these antagonists in G<sub>0</sub>/G<sub>1</sub> and S phases, respectively. However, we cannot rule out the involvement of other cell cycle regulators, such as p27 (20). Our results also suggest that two mediators, [Ca<sup>2+</sup>], and cAMP, control progression through G<sub>0</sub>/G<sub>1</sub> and S phases of the cell cycle, respectively, by PGE<sub>2</sub> interaction with EP<sub>1</sub> and EP<sub>4</sub> subtype receptors.

We thank Robin Rycroft for valuable assistance in the preparation of the English manuscript.

This study was supported by the Spanish Ministry of Education (PM97-0110 and PM98-0191). T. Sanchez was a recipient of a doctoral fellowship from the Autonomous Government of Catalonia.
FACS analyses were performed at the Serveis Científico-Tècnics, University of Barcelona, Barcelona, Spain. AH-6809 and AH-23848B were kindly provided by Dr. S. G. Lister from the Compound Supplies Officer, Glaxo Wellcome, Medicines Research Centre, Stevenage, UK.

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