Effect of indomethacin on cell cycle dependent cyclic AMP fluxes in tobacco BY-2 cells

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Abstract The evolution of adenosine 3′,5′-cyclic monophosphate (cAMP) levels was investigated in synchronised tobacco BY-2 cells by virtue of a method based on immunoaffinity purification and analysis on electrospray tandem mass spectrometry. A transient peak in cAMP content was observed during the S and G_1 phases of the cell cycle. Application of the prostaglandin inhibiting drug indomethacin at early S phase resulted in the loss of the cAMP peak in S phase and inhibited mitotic division. This inhibition of cAMP accumulation suggests the presence of a prostaglandin-dependent adenylyl cyclase activity, analogous to animal cyclases. A potential role for cAMP during the plant cell cycle is postulated.

Key words: Cell cycle; Adenosine 3′,5′-cyclic monophosphate; Indomethacin; Mass spectrometry; Synchronisation; Tobacco BY-2 cell

1. Introduction

Adenosine 3′,5′-cyclic monophosphate (cAMP) is an important regulatory molecule in a vast multitude of cellular processes. For example, cAMP has been implicated in catabolite repression [1], chemotaxis [2], and hormone action [3]. Cyclic AMP is thought to play a role in cell cycle control in animal and fungal systems. Its concentration fluctuates during cell cycle progression [4] and depending on the cell type it exhibits stimulatory or inhibitory effects on cell proliferation [5,6]. A transient rise in cAMP before the onset of S phase is believed to be part of a series of events leading to DNA synthesis [7]. Cyclin A, cyclin D and cyclin E expression are affected by cAMP [8–11]. A direct phosphorylation of cyclin D_1 in the cyclin box by a cAMP-dependent protein kinase is thought to regulate its activity [12]. Forskolin and 8-Br-cAMP inhibit cyclin A- and cyclin E-dependent histone H1 kinase activity in an astrocytic cell line [11]. The *Saccharomyces cerevisiae* cell cycle is highly regulated by the RAS/cAMP signal transduction pathway as well [13,14]. Cyclic AMP was shown to be a key regulator of the circadian rhythm driven cell cycle of the unicellular alga *Euglena gracilis* [15,16].

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Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; BY-2, bright yellow 2; LC, liquid chromatography; MS, mass spectrometry; RP, reversed phase; MRM, multiple reaction monitoring; MeOH, methanol; HAc, acetic acid; Aφ, aphidicolin; PZ, propyzamide; IM, indomethacin

Data on the presence of cAMP and its metabolism in vascular plants are continuously coming forth. However, the physiological importance of cAMP in plants remains a matter of debate [17–19]. The plant cell cycle seems mechanistically similar to that of other eukaryotes and most of the key regulators of the animal cell cycle are conserved in higher plants [20–24]. This prompted us to investigate a comparable function for cAMP in the cell cycle of higher plants.

As an experimental system tobacco BY-2 cells were chosen because of their high growth rate, homogeneity and synchronisation potential [25]. The role of cAMP in cell cycle progression was studied by addition of indomethacin, a potent inhibitor of prostaglandin synthesis [26] reported to inhibit adenylyl cyclase in animal cells [27]. In contrast to animal systems where the relationship between indomethacin treatment and cAMP metabolism has been well studied, its inhibitory action on both cAMP synthesis and cell cycle, as described in this paper, has never been reported in plant cells before.

2. Materials and methods

2.1. Culture maintenance, synchronisation and sampling

Tobacco BY-2 cell cultures were maintained as described by Nagata et al. [25]. Depending on the aim of the experiment either an aphidicolin block or a sequential aphidicolin–propyzamide block was performed. Ten to fifteen ml of stationary culture were transferred to 100 ml of MS medium containing 5 μg/ml aphidicolin (Sigma). After 24 h cells were extensively washed and released in 100 ml of fresh medium. Unless stated otherwise aphidicolin block was used as the default synchronising tool. Propyzamide (1.54 μg/ml) was added during G_2 phase (about 4 h after aphidicolin release) and removed by extensive washing when almost all cells were synchronised in prometaphase.

2.2. Mitotic index, Northern analysis, nuclei isolation and flow cytometry

Samples for mitotic index, Northern analysis and flow cytometric analysis were gathered every hour. To determine the mitotic index, cells were fixed with ethanol/acetic acid (3:1, v/v) and stored at 4°C until analysis (within 24 h). Fixed cells were stained with 5 μM DAPI (4′,6-diamidino-2-phenylindole) and analysed using a fluorescent microscope (Leitz, Germany) by counting the number of nuclei (300 cells) in late prophase to telophase. Viability tests were done by adding FDA (fluorescein di-acetate) at a concentration of 5 μg/ml cell suspension culture.

Total RNA was prepared using the TRIzol reagent (Gibco/BRL) according to the manufacturer's instructions. RNA blots were hybridised at 65°C in 500 mM NaPi buffer containing 7% SDS, 1% BSA and 1 mM EDTA [28] to random primed ^{32}P probes corresponding to the coding region of the *Arabidopsis thaliana* H4A748 gene [23]. Transcript levels were quantified from the blots using a PhosphorImager (Molecular Dynamics).

Nuclei were purified from the cells by enzymatic treatment accord-

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ing to [29] and stored at 4°C until further analysis by flow cytometry. On the day of analysis, isolated nuclei were treated with RNAse A, stained with propidium iodide (50 μg/ml) and analysed with a FACS scan flow cytometer.

2.3. Extraction and purification of cAMP

Samples for cAMP determination were collected every 30 min. The cells were left to sediment on ice and the supernatant was carefully removed. Samples were frozen in liquid nitrogen and kept at −80°C until analysis. Seven to eight hundred mg of cells were ground with a mortar and pestle in liquid nitrogen and homogenised in an ice-cold mixture of methanol/chloroform/1 M formic acid (12:5:3; v/v) containing 1000 Bq [^{3}H-2′,8]-3′,5′-cAMP as an internal standard. The samples were immunoaffinity-purified according to Roef et al. [30] and stored at −20°C until further analysis.

2.4. Cyclic AMP identification and quantification

Immunoaffinity-purified samples were dissolved in 25 μl of the mobile phase and injected into the LC-UV-MS/MS combination consisting of a Kontron 465 HPLC Autosampler, the Kontron 325 HPLC pump, the Kontron 332 UV-detector (Kontron Instruments, Italy) and the Quattro II tandem mass spectrometer (Micromass, UK). Samples were eluted using ion suppression conditions (0.25% HAc, 9% MeOH, pH 3.5) on a microbore HPLC column (C18 Adsorbosphere, 250×2.1 mm, Alltech Associates Inc., Belgium) using a flow rate of 200 μl/min. Electrospray mass spectra were recorded in negative ionisation mode with the probe tip kept at −3 kV, a cone voltage of 32 V and a source temperature of 125°C. Data were interpreted using the accompanying MassLynx Mass Spectrometric Data Handling System for Windows (Micromass, UK). Quantification was carried out by multiple reaction monitoring (MRM) using the diagnostic transition 328→134 [31]. Final cAMP concentrations in plant cells were calculated from the area of the MRM ion chromatogram. They were expressed as picomole 3′,5′-cAMP per gram of fresh weight of BY-2 cells.

3. Results

3.1. Cyclic AMP levels in synchronised TBY-2 cells

Cyclic AMP content was measured in aphidicolin synchronised tobacco BY-2 cells (Fig. 1, upper part). Cell cycle progression was monitored by determination of mitotic index, histone 4 (H4) gene expression and flow cytometry (Figs. 1 and 3). BY-2 cell suspensions synchronised with aphidicolin generally show a peak in mitotic index of about 40% at 6 to 7 h after release. By 9 to 10 h of culture all cells have reached G_1 phase (Fig. 3, t_0 Aφ through t_{10} Aφ). Cyclic AMP levels showed distinct peaks during S phase (maximum H4 mRNA expression) and G_1 phase (Fig. 1, upper part). No additional peaks above a calculated detection limit of approximately 5 pmol/gfw appeared at other stages of the cell cycle.

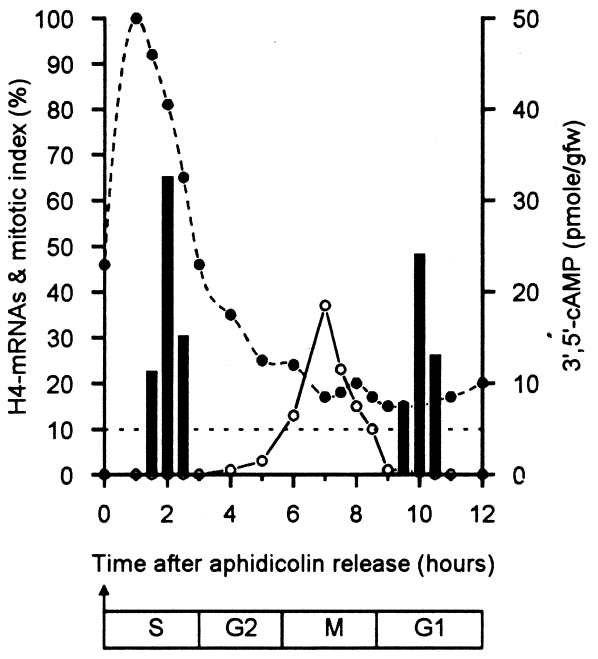

Fig. 1. Cyclic AMP content (solid bars) in aphidicolin synchronised cells (dashed line represents the detection limit). ○—○: Mitotic index; ●- - -●: H4-mRNA levels. The approximate duration of the different phases of the cycle is represented in the lower part (↑: Aφ release).

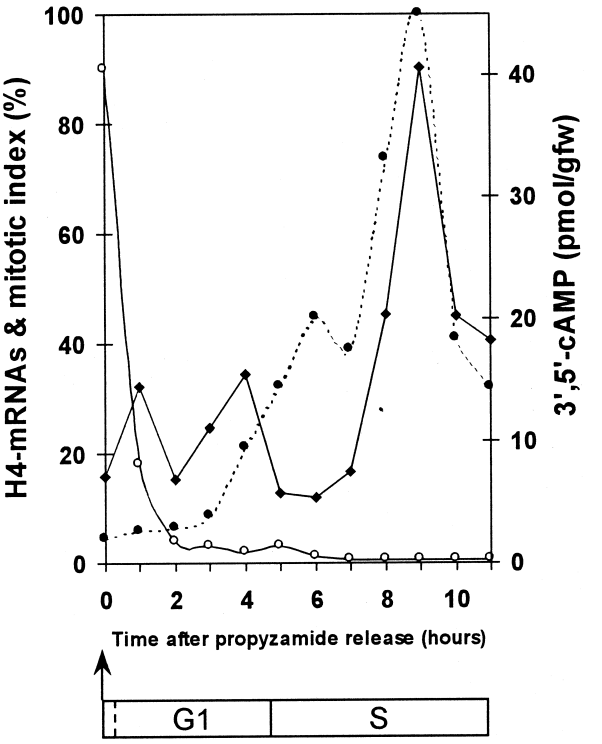

Fig. 2. Cyclic AMP content (♦—♦) in aphidicolin-propyzamide synchronised cells. ○—○: Mitotic index; ●- - -●: H4-mRNA levels. The approximate duration of the different phases of the cycle is represented in the lower part (↑: PZ release).

Cyclic AMP fluxes during cell cyclic were further documented in cells that were sequentially treated with aphidicolin and propyzamide (Fig. 2). The sequential aphidicolin-propyzamide block resulted in a high synchronisation at prometaphase with a mitotic index reaching 90% (Fig. 2). H4 mRNA steady state levels were most abundant 9 h after propyzamide release. Cells synchronised by the double aphidicolin-propyzamide block again showed elevated cAMP levels in S phase coinciding with H4 mRNA expression (Fig. 2). The rise in cAMP noticed during G_1 phase in aphidicolin-treated cells was less apparent here.

3.2. Indomethacin blocks BY-2 cells at G_2 phase of the cycle

The influence of indomethacin on tobacco BY-2 cell cycle was investigated in cells supplied with 10 μg indomethacin/ml immediately after aphidicolin release. Cell cycle progression was analysed by flow cytometry (Fig. 3) and mitotic index (Fig. 4a). DNA content distribution revealed cells passed through S phase to be subsequently retained in G_2 (Fig. 3: t_1 IM through t_7 IM) whereas control cells progressed

through S-G2-M and eventually reached G1 (Fig. 3, lane 1; Fig. 4a). Some indomethacin treated cells eventually showed mitosis 9 h after aphidicolin release and passed to G_1 phase

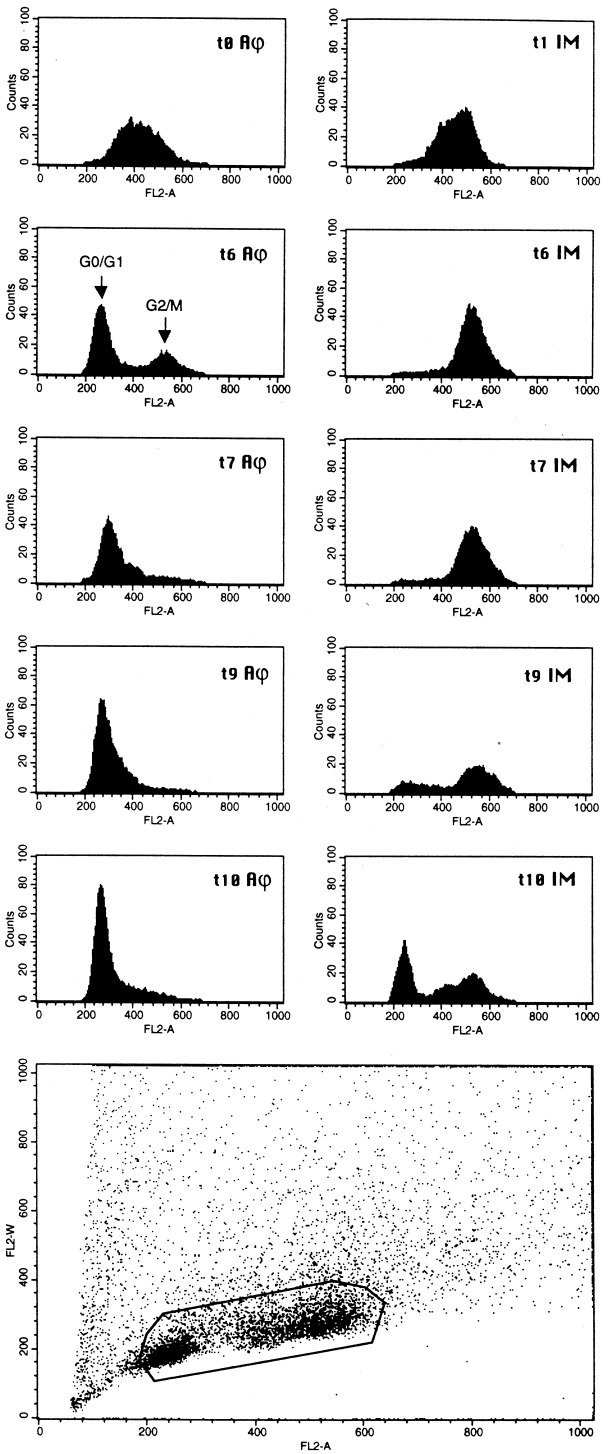

Fig. 3. Flow cytometric analysis of DNA content in indomethacin treated cells. Lane 1: control cells; lane 2: IM treated cells. The dot plot top represents a cytogram of the width vs. area fluorescence of the DNA signal of an indomethacin treated sample (t_{10} IM). Aggregates were excluded from real G_2/M phase nuclei by fluorescence pulse shape analysis, based on the fact that aggregates exhibit a larger pulse width. The trail of low fluorescence signals towards the origin represents nuclear debris. Only dots within the region displayed are included in the histogram analysis.

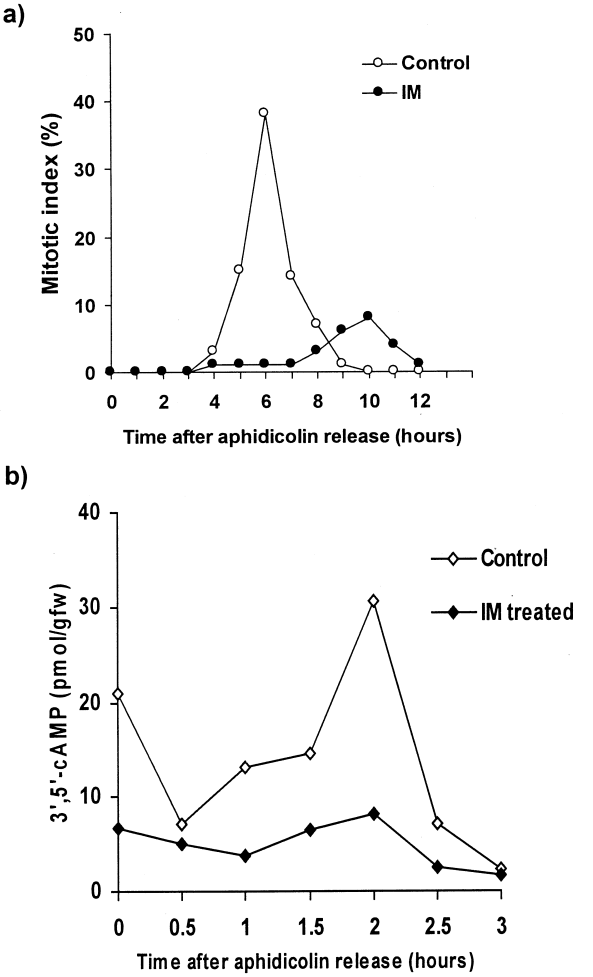

Fig. 4. a: Mitotis in indomethacin treated cells. ○—○: Control cells; ●—●: indomethacin treated cells. b: Inhibition of cAMP accumulation by indomethacin during S phase. ◇—◇: Control cells; ◆—◆: indomethacin treated cells.

(Fig. 3: t_9 IM and t_{10} IM; Fig. 4). The fact that indomethacin treated cells eventually do engage in mitosis and escape into G_1 suggests that indomethacin was not toxic at the concentrations used. This observation was confirmed by viability staining (data not shown).

3.3. Inhibition of cAMP accumulation by indomethacin

In animal systems, indomethacin acts on the cell cycle through the indirect inhibition of adenylyl cyclase activity [27]. We therefore investigated the behaviour of cAMP accumulation during S phase as a response to indomethacin addition. Our results show that the S-phase associated rise in cAMP was completely abolished in cells treated with indomethacin (Fig. 4b). Non-treated cells showed a marked increase in cAMP content during S phase with peak values of approximately 30 pmol/gfw at 2 h after release. In cells treated with indomethacin these levels were reduced to values of around 5 to 7 pmol per gram fresh weight. Since indomethacin interferes with mitosis as well, a potential role for cAMP in cell cycle progression in plant systems is postulated. In order to determine the necessity of cAMP for proper cell cycle progression, cAMP was delivered to indomethacin-treated cells and cell cycle progression and mitotic division were ex-

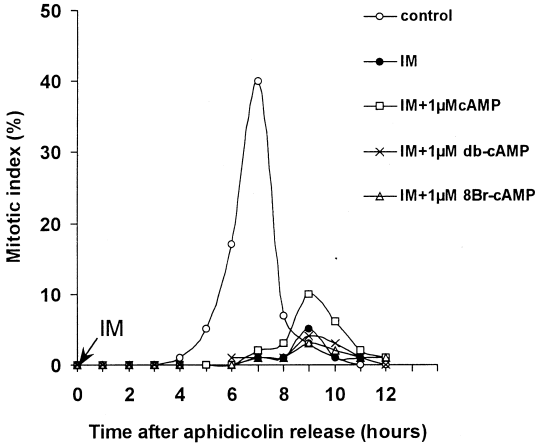

Fig. 5. Mitotis in indomethacin treated cells. Salvage by cAMP and cAMP-analogues.

amined. The addition of cAMP to indomethacin-inhibited cells failed to restore mitosis. The addition of more membrane-permeable cAMP analogues (8-Br-cAMP and dibutyryl-cAMP) could not rescue indomethacin-arrested cells either (Fig. 5).

4. Discussion

The evolution of cAMP levels during the plant cell cycle was studied in tobacco BY-2 cells synchronised by means of aphidicolin treatment or sequential aphidicolin-propyzamide block. Two important observations were made. It appears that cAMP levels are stringently controlled during the tobacco BY-2 cell cycle and that both cAMP accumulation and mitosis are inhibited upon indomethacin treatment.

Our data show that fluctuations in intracellular cAMP levels of synchronised tobacco BY-2 cells are tightly connected to cell cycle progression. Cyclic AMP peaks were observed in S phase, and to a lesser extent in G_1 phase, in cells synchronised with either the aphidicolin or aphidicolin-propyzamide method. As these cAMP peaks were observed at the same stages of the cell cycle in both synchronisation systems, they are genuinely cell cycle dependent and not, for instance, stress induced.

This is to our knowledge the first time a cell cycle regulated cAMP accumulation is shown in a higher plant. Cell cycle dependent fluctuations of cAMP have been observed in a number of animal systems [4,7,32] and fungi [13,14]. Generally, a peak in cAMP is observed during G_1 phase. This peak is believed to be involved in the initiation of DNA synthesis. A second peak during G_2 seems to be correlated to the onset of mitosis [32]. Similar surges in cAMP in G_1 and at the onset of mitosis were observed in the unicellular alga *Euglena gracilis*. They are believed to synchronise cell division of this lower plant with circadian rhythm by guiding cells through G_1/S and G_2/M boundaries [33]. The fluctuations in cAMP levels we observed during the tobacco BY-2 cell cycle are very reminiscent of the ones encountered in mastocytoma P-815 cells [4], with peak values of cAMP in S phase and G_2 phase, and to a lesser extent in G_1. The peaks at S and G_2 are probably induced by PGE_1. Although the significance of the peak at S phase is unclear the tight control of cAMP levels during tobacco BY-2 cell cycle strongly argues for an important role of cAMP in cell cycle progression in higher plants.

Since an indomethacin-induced inhibition of mitosis was recognised in our TBY-2 cells, this study is to our knowledge the first to show such susceptibility of a plant system to indomethacin. In lymphocytes the indomethacin-induced arrest of the cell cycle is caused by the inactivation of a prostaglandin-induced adenylyl cyclase activity [27]. A similar relationship between indomethacin and cAMP metabolism seems to exist in tobacco BY-2 cells. The observed inhibition of cAMP accumulation in response to indomethacin treatment is indeed indicative for the presence of a prostaglandin-dependent adenylyl cyclase activity in tobacco BY-2 cell. Such connection has never been reported before for plant systems. The mere existence of prostaglandin metabolism and definitely cAMP metabolism in plants is actually not widely accepted yet [17,34]. Addition of prostaglandins, or prostaglandin-like compounds, to indomethacin blocked cells and subsequent analysis of cAMP content and adenylyl cyclase activity should provide clues as to whether the same signal transduction pathway is active in the plant cell.

The observation that indomethacin simultaneously prevents cAMP accumulation and inhibits mitosis in the tobacco BY-2 system strengthened our belief in the need for cAMP for proper cell cycle progression. However, experiments set up to rescue indomethacin blocked cells by addition of exogenous cAMP failed. This cannot be explained by poor uptake of cAMP into the cells. Data not shown in Section 3 indicated that ^{3}H-cAMP, when supplied to the culture medium at a concentration of 1 μM, did accumulate in the cells to an extent were it is able to perform its action. In addition, dibutyryl-cAMP and 8-Br-cAMP were equally inadequate in rescuing indomethacin-blocked cells. We therefore conclude that an increase in intracellular cAMP alone cannot counteract the indomethacin block. Many reports suggest that the cAMP peak at G_1/S must be transient to allow for proper cell cycle progression in animal cells. A rise in cAMP is necessary for the onset of DNA synthesis but the persistent elevation of cAMP supposedly blocks initiation of S phase or prematurely terminates DNA replication [7,32]. This was also shown for *Euglena gracilis*. Sustaining the G_1 peak by addition of exogenous cAMP caused a delay in the progression of cells through S phase [33]. The failure to rescue indomethacin blocked cells by addition of cAMP might reflect the presence of a similar mechanism in tobacco cells.

In animal systems, indomethacin is a pleiotropic drug acting primarily on prostaglandin synthesis, and consequently on adenylyl cyclase. In this report we have provided evidence indicative for the presence of a similar pathway in TBY-2 cells. But in some instances indomethacin appears to act downstream from cAMP too by directly affecting cAMP-dependent protein kinase activity [35]. In addition, indomethacin might also exert an effect on cell cycle following from its impact on signal transduction pathways branching out from other prostaglandin regulated events (with cyclic AMP just being one of the second messengers affected). In both cases the sole addition of cAMP will prove ineffective in restoring indomethacin induced inhibition of mitosis. Evidence for a positive need of cAMP for proper cell cycle progression should therefore come from experiments in which cAMP accumulation is inhibited by means of methods independent of prostaglandin metabolism. At present, the tools to do so are

not at hand. Chemicals exist that inhibit adenylyl cyclase activity in animal systems but none of them have been adequately characterised in plant systems. Future work will pursue the identification of such compounds and their influence on the plant cell cycle.

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