Indomethacin-induced G1/S phase arrest of the plant cell cycle

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Abstract In animal systems, indomethacin inhibits cAMP production via a prostaglandin-adenylyl cyclase pathway. To examine the possibility that a similar mechanism occurs in plants, the effect of indomethacin on the cell cycle of a tobacco bright yellow 2 (TBY-2) cell suspension was studied. Application of indomethacin during mitosis did not interfere with the M/G1 progression in synchronized BY-2 cells but it inhibited cAMP production at the beginning of the G1 phase and arrested the cell cycle progression at G1/S. These observations are discussed in relation to the putative involvement of cAMP biosynthesis in the cell cycle progression in TBY-2 cells.

Key words: Indomethacin; Propyzamide; Tobacco bright yellow 2 cell; Cell cycle; Synchronization; Cyclic AMP

1. Introduction

Fluctuations of cyclic AMP (cAMP) concentrations represent an important means of signal transduction during the cell cycle progression in animals and yeast. Depending on the cell type, fluctuation in cAMP levels can lead to either stimulatory or inhibitory effects [1–3]. In yeast, the Ras/cAMP [4–6] pathway plays a central role in co-ordinating cell growth and metabolism. Cells deficient in cAMP dependent protein kinase (PKA) arrest in G1 [4]. In an astrocytic cell line, cAMP inhibits cyclin dependent kinase (CDK)-cyclin complexes in the G1 phase of the cycle [7].

Data on the presence of cAMP in higher plants are continuously emerging since 25 years, but the functional role of this molecule is still poorly defined and as a result, the 'cAMP controversy' remains unresolved [8–12]. In the lower plant *Euglena gracilis*, cAMP and its metabolic components were shown to be key regulators of the circadian rhythm driven cell cycle [13,14]. In the embryos and seedlings of *Haplopappus gracilis*, inhibition of the cAMP degradative enzyme phosphodiesterase by aminophylline results in a cell cycle block in the G1 and G2 phase [15].

In a tobacco bright yellow 2 (TBY-2) cell suspension, culture accumulation of cAMP has been shown to correlate with the S and G1 phase of the cell cycle [16]. Furthermore, it was shown that the addition of indomethacin, which has been extensively studied as an inhibitor of prostaglandin dependent adenylyl cyclase and cAMP dependent protein kinase activity in animal cells [17–20], simultaneously prevents the accumulation of cAMP in the S phase and arrests the cells in the G2/M phases of the cycle [16]. These findings were in accordance with data showing that in lymphocytes, indomethacin arrests DNA synthesis by the inactivation of adenyl cyclase activity [18], whereas in rat hepatoma cells and human diploid fibroblast cells, addition of indomethacin blocked the cell cycle at the G1 phase [21,22].

Highly synchronous cell populations are required to study the regulatory events of the cell cycle. From this point of view, the TBY-2 cell suspension culture provides valuable advantages in plant cell cycle research because of its fast growth rate and high synchronization potential [23]. By using different cell cycle blocks as a tool for synchronization, this cell suspension culture offers unique opportunities to study the genetic and biochemical events of the plant cell cycle. In this paper, we report on the effect of indomethacin, when exogenously applied during mitosis of propyzamide-synchronized BY-2 cells, on both the inhibition of cAMP biosynthesis in G1 and progression through M, M/G1, G1 and arrest in G1/S.

2. Material and methods

2.1. TBY-2 synchronization and sampling

TBY-2 cell cultures were maintained as described by Nagata et al. [23]. In brief, 1.5 ml of stationary culture was transferred to 100 ml of MS medium (30 g/ml sucrose, 0.2 g/ml KH_2PO_4, 4.302 g/ml MS) and cultured for 7 days. Depending on the aim of the experiment, either a sequential aphidicolin-propyzamide or an aphidicolin-oryzalin block was performed. 10–15 ml of stationary culture was transferred to 100 ml of MS medium containing 5 μg/ml aphidicolin (ICN). Aphidicolin-treated cells were extensively washed and released in 100 ml of fresh medium after 24 h. In this synchronization system, parallel studies were performed by treating cells with indomethacin at a concentration of 10 μg/ml and indomethacin+aphidicolin for 24 h in order to compare the effect of indomethacin with aphidicolin. To observe the effect of indomethacin on the M to G1 transition, we delivered indomethacin (10 μg/ml) at the time of the mitotic peak, 6 h after the cells were released from aphidicolin. Samples were collected every hour in order to perform mitotic index and flow cytometric measurements.

In a first attempt to obtain a higher synchronization, we applied oryzalin (15 μM) at the end of the G2 phase in aphidicolin-released cells [24]. Because of experimental problems related to the use of oryzalin (see Section 3), we later delivered propyzamide (1.54 μg/ml) during the G2 phase (about 4 h after aphidicolin release). When almost all cells were synchronized in the prometaphase [16], propyzamide was removed by extensive washing. To observe the effect of indomethacin on the G1 and G1/S phase, one part of propyzamide-released cells was treated with indomethacin (1.54 μg/ml) while the other part was not treated. Samples were collected every hour for

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Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; TBY-2, tobacco bright yellow 2; CDK, cyclin dependent kinase

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mitotic index, Northern analysis and flow cytometric analysis and cAMP analysis. 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.

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

Fixed cells (ethanol:acetic acid, 3:1, v/v) were stained with 5 μM 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes, USA) and analyzed using a fluorescent microscope (Leitz, Germany) by counting the number of nuclei (300 cells) in the late prophase to telophase. Fluorescein diacetate (5 μg/ml) was used for viability staining. Total RNA was prepared according to the method described by Goodall et al. [25]. RNA blots were hybridized at 65°C in a phosphate buffer in the presence of 50% formamide to random-primed ^{32}P probes corresponding to the coding region of the *Arabidopsis thaliana* H4A748 gene and cyclin A59 gene [26,27]. For nuclei purification, we conducted enzymatic treatment according to [28]. Samples were 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 analyzed with a FACS scan flow cytometer.

2.3. cAMP extraction, purification and quantification

For cAMP extraction and purification, we followed the method described in Ehsan et al. [16] with little modification. In brief, 200–400 mg of cells was ground with a mortar and pestle in liquid nitrogen and homogenized in an ice-cold mixture of methanol:chloroform:1 M formic acid (12:5:3, v/v/v) containing 585 Bq [^{3}H]2′,8-3′,5′-cAMP as an internal standard. The samples were immuno-affinity-purified according to Roef et al. [29] and stored at −20°C until further analysis. Immuno-affinity-purified samples were quantified according to the method described in Witters et al. [30].

3. Results

3.1. Progression from the M to G1 phase is not affected by indomethacin

In an attempt to establish whether indomethacin has an effect during the transition from M to G1, we applied indomethacin at the time of the mitotic peak (6 h) in aphidicolin-released cells (Fig. 1). In the control culture, cells progressed from the M phase to G1 in 1 h. Indomethacin-treated cells proceeded at a comparable rate (Fig. 1a), although the flow cytometric analysis showed that some cells remain in the G2/M phase of the cycle. These are probably cells from a population that is still in S or G2 at the time of application of indomethacin [16]. From MI measurements (Fig. 1b), it can be seen that cells that were engaged in mitosis proceeded to the G1 phase. Thus, indomethacin showed no inhibitory action on M/G1 progression.

3.2. G1/S phase arrest in indomethacin-treated BY-2 cells

Freshly diluted stationary cells, which were in G1 (Fig. 2a), were withheld in G1 for 96% after a 24 h treatment with indomethacin (10 μg/ml) (Fig. 2c). Aphidicolin treatment for 24 h resulted in S phase arrest (Fig. 2b). Addition of aphidicolin+indomethacin for 24 h resulted in G1 block (Fig. 2d) as was the case when only indomethacin was added (Fig. 2c). This shows that indomethacin induces cell cycle arrest before the aphidicolin block in S.

The synchronicity of aphidicolin-released cells was not high enough (MI = 30–50%) to study a second cell division cycle. Therefore, we blocked the cells for 24 h with aphidicolin. Subsequently, we applied different M phase blocks to these aphidicolin-released cells (Fig. 3). When we delivered oryzalin (which inhibits microtubule polymerization) at a concentration of 15 μM, almost 90% cells blocked at the metaphase

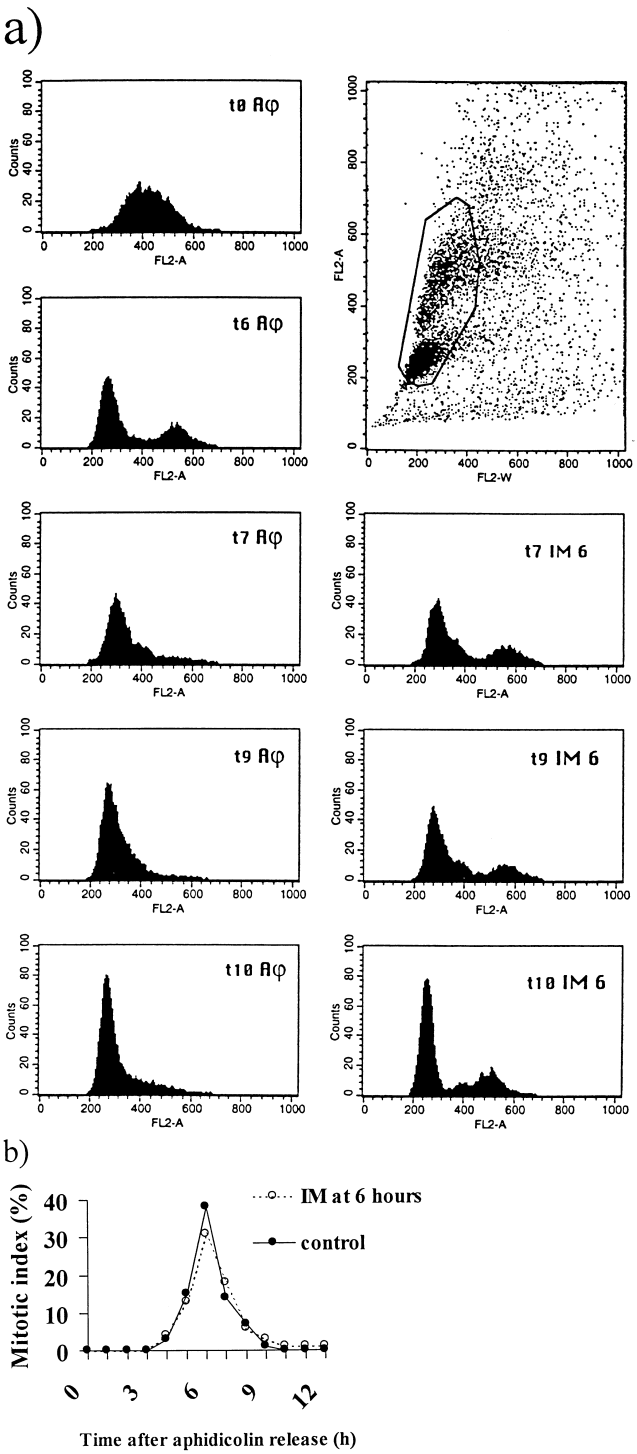

Fig. 1. Effect of indomethacin on M/G1 progression. Indomethacin was delivered at the time of the mitotic peak (6 h) in aphidicolin-released cells. Cell cycle progression was determined by flow cytometric analysis (a, left: control cells; right: indomethacin-treated cells) and the mitotic index (b). The dot plot (top right) represents a cytogram of the width versus area fluorescence of the DNA signal of indomethacin-treated cells. Only dots within the region displayed are included in the histogram analysis.

(Fig. 3a). When we removed this drug by washing in order to observe the progression from the M to G1 phase of the cycle, major difficulties arose. About 25% of the cells were still in the M phase 2 h after oryzalin release and more than 10%

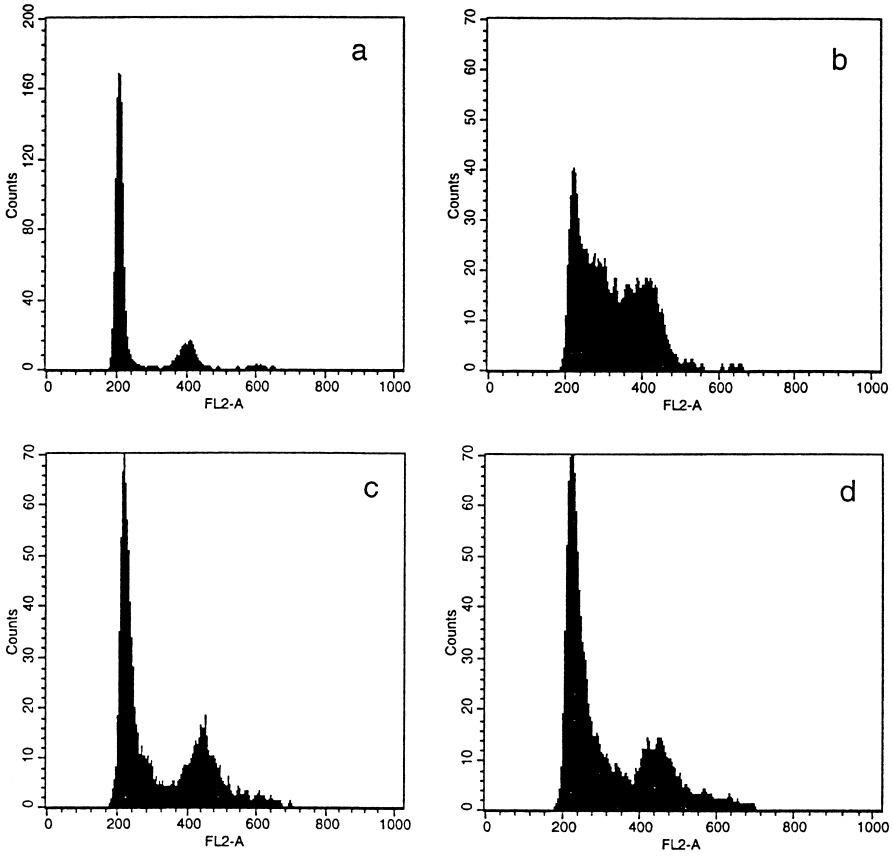

Fig. 2. Flow cytometric analysis of the DNA content in indomethacin/aphidicolin-treated cells. a: Control cells in a stationary culture; b: aphidicolin-treated cells; c: indomethacin-treated cells and d: indomethacin+aphidicolin-treated cells.

remained in M for at least 8 h (Fig. 3a). Compared to control cells, the size of the nuclei was considerably larger (Fig. 3b) and most important, cells were not amenable to flow cytometric analysis. No such problems were encountered when propyzamide (which also inhibits microtubule polymerization) was used. Upon application of this compound at a concentration of 1.54 μg/ml to aphidicolin-released cells, we obtained 90% synchronization (metaphase block). All of these cells reached the G1 phase within 2 h after washing off the propyzamide (Fig. 3a). We added indomethacin to these highly synchronized aphidicolin/propyzamide-released cells to observe whether indomethacin has any effect on the progression through the G1 and S phase of the cycle. The indomethacin-treated cells proceeded from the M to G1 phase in the same fashion as the control cells did, but remained blocked in G1 (Fig. 4a). The expression of the S phase markers histone H4 and cyclin A59 showed a clear G1/S block in indomethacin treated cells (Fig. 4b). Viability tests in indomethacin-treated cells showed no significant differences compared to the control cells (data not shown).

3.3. Inhibition of cAMP accumulation by indomethacin

A fluctuation of cAMP levels during the cell cycle is a part of the series of events for proper cell cycle progression in animal systems [18]. In this study, we investigated whether the G1/S block by indomethacin is related to inhibition of cAMP accumulation in the G1 phase. Therefore, we analyzed cAMP concentrations in indomethacin-treated cells at different time points in the G1 phase. Our results show that in control cultures, an accumulation of cAMP was observed whereas in indomethacin-treated cells, cAMP accumulation was inhibited at the beginning of G1 (Fig. 5). The cAMP peak in the control culture corresponds to 24 pmol/gfw at this time point.

4. Discussion

TBY-2 cell suspension cultures have been extensively used during the last few years for plant cell cycle analysis. This highly synchronizable cell line has not only facilitated the dissection of the molecular machinery of the plant cell cycle but also the identification of the effect of different compounds on it. An interesting finding in the present study is the effect of oryzalin on this BY-2 cell line where perhaps nuclear polyploidy occurs. However, our objective was to reach a higher synchronization and we therefore proceeded our investigation by using propyzamide. In contrast to oryzalin-treated cells, propyzamide-exposed cells showed no abnormal morphological changes.

The role of regulatory components during plant cell cycle progression is still much less understood compared to animal cells [31,32]. In all eukaryotes, two important control points exist (G1/S and G2/M) where CDK-cyclin complexes are major phosphorylation players. The G1/S is a key regulatory point where the cell decides whether or not to enter the DNA replication step. Hence, molecules that inhibit G1/S phase progression have been considered to be excellent candidates not only for controlling the cell cycle but also to dissect

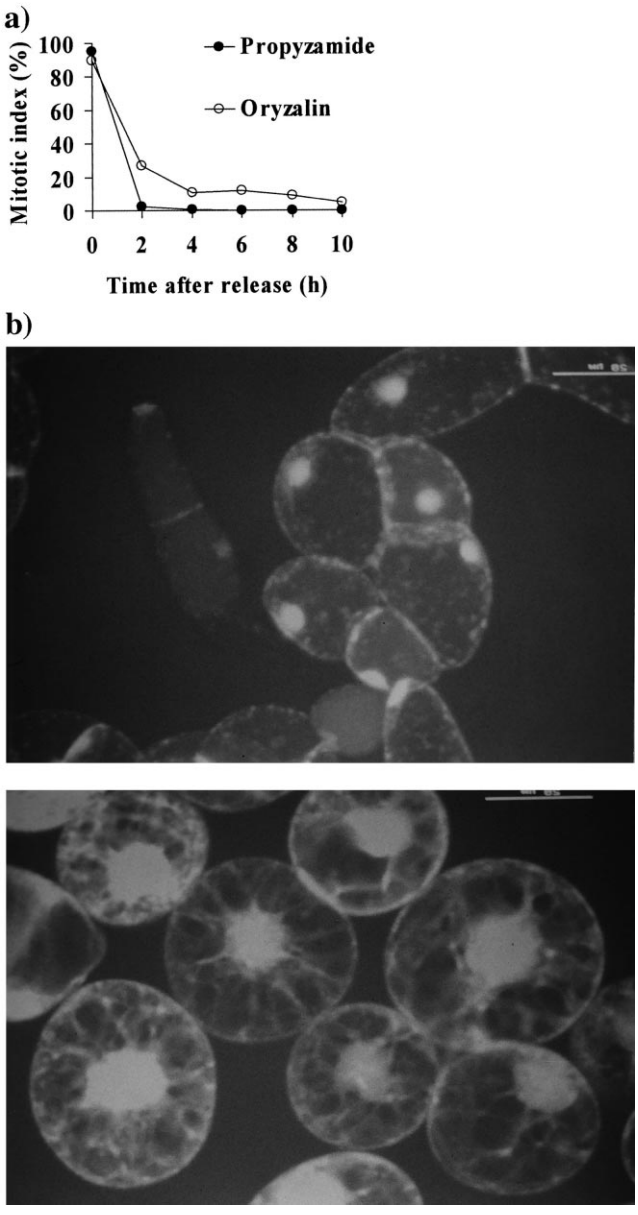

Fig. 3. Effect of oryzalin in TBY-2 cells. a: Mitotic index of oryzalin- and propyzamide-treated cells. b: Light microscopic analysis of oryzalin-treated cells (upper) and control cells (lower). Scale bar indicates 50 μm.

the molecular mechanism underlying this important phase. Northern analysis of histone H4, cyclin A expression levels and flow cytometric analysis of indomethacin-treated cells gave a clear picture of G1/S inhibition. This demonstrates that besides the described reversible inhibition of growth of animal cells in the G1 phase [21,22], indomethacin also blocks TBY-2 cells at the G1/S phase. The addition of indomethacin during the M phase did not retard the progression from the M/G1 phase of the cycle. This is in contrast with animal cells, where the cAMP/PKA pathway is necessary for M/G1 transition [33]. Our observation is supported by our previous study where no cAMP peak was detected during the M phase of the cycle in aphidicolin-released TBY-2 cells [16]. The present study shows, for the first time, that indomethacin inhibits plant cell cycle progression in the G1/S phase. Still, its functional pathway needs to be documented.

Our findings not only demonstrated the effect of indomethacin during the cell cycle but also fluctuations of plant cAMP during the cell cycle progression. cAMP is involved

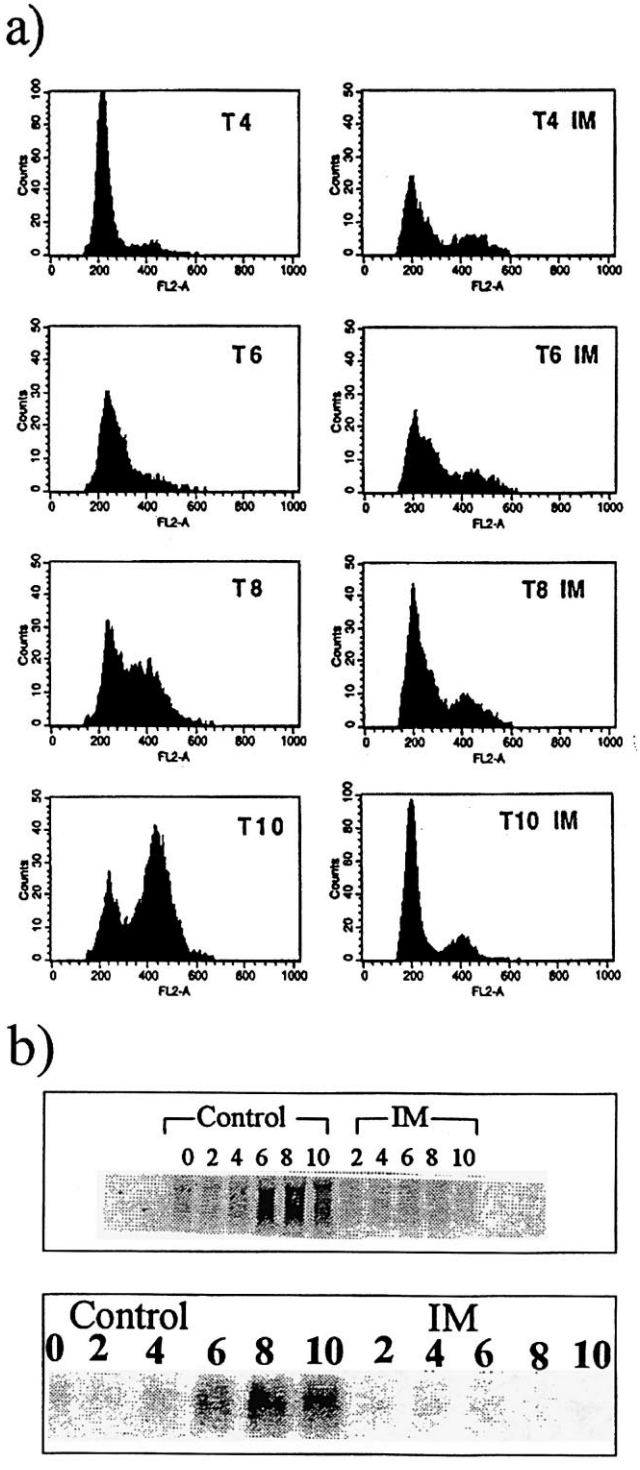

Fig. 4. a: Flow cytometric analysis of indomethacin-treated cells at 2 h intervals (T4-T10). Left: control cells (aphidicolin/propyzamide-released). Right: indomethacin-treated cells (aphidicolin/propyzamide-released). b: Northern analysis of cyclin A (upper) and H4 (lower) expression levels in control and indomethacin-treated cells at 2 h intervals (0–10) after aphidicolin/propyzamide release.

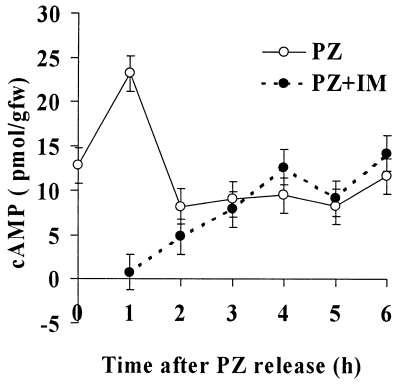

Fig. 5. cAMP content of aphidicolin/propyzamide-released (solid line) and aphidicolin/propyzamide-released indomethacin-treated cells (dashed line).

in the regulation of a wide variety of developmental processes in animal and yeast. In these systems, cAMP either blocks or promotes cell cycle progression in the mid to late G1 phase [1,34,35]. For example, it was demonstrated that PKA phosphorylates cyclin D1 at Ser-90 in the cyclin box and induces p27 (CDK inhibitor) in an animal system [36,37]. Induction of cyclin A in the restriction point by cAMP and suppression by PKA inhibitors has been reported in human fibroblast cell lines [38]. In our investigation, the oscillation of cAMP at the beginning of the G1 phase as well as its inhibition by indomethacin raised intriguing questions for the possible involvement of cAMP for proper cell cycle progression. Although not known, we can assume that the effects of indomethacin on cAMP production at the beginning of the G1 phase affect the complex mechanism by which cells blocked at the G1/S phase of the cycle.

In higher plants, the putative involvement of cAMP during the plant cell cycle is largely unknown. However, there is increasing evidence of the involvement/fluctuation of cAMP during the cell cycle from lower to higher plants. PKA has not yet been clearly documented but other components involved in cAMP action are described in plant cells. For example, putative cAMP response element binding proteins and an adenylyl cyclase-associated protein have been identified in higher plants [39,40]. Here, we provide data for the presence of cAMP in the G1 phase and the inhibition of cAMP in the beginning of the G1 phase by indomethacin. This strengthens our believe in the necessity of cAMP for proper cell cycle progression.

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