Plant Tissue Cult. **14**(1) : 69-74, 2004 (June)

PTC

Cyclic AMP in Auxin Signal Transduction Pathway ?

Hashimul Ehsan* and Harry Van Onckelen[1]

Plant Biology Department, University of New Hampshire, Durham, NH-03824, USA

Key words: Tobacco BY-2 cell, Cell cycle, Synchronisation, Cyclic AMP, Auxin

Abstract

The putative involvement of cAMP in downstream events of the auxin signal transduction pathway was studied in aphidicolin synchronised tobacco BY-2 cells. We applied cAMP analogues either to 2-NAA inhibited or to auxin starved tobacco BY-2 cell cultures with the idea that cAMP could rescue mitotic division by triggering an auxin response. None of the applied cAMP analogues induced mitotic division in either of the two experimentally inhibited tobacco BY-2 systems.

Introduction

The necessity of auxin for cell division is well documented; however, a clear picture of the downstream steps (second messengers, kinase cascade, etc.) of auxin during the cell cycle has yet to emerge. Thus far, the isolation of an auxin responsive gene that encodes a protein structurally related to the beta-subunit G-protein (Ishida et al. 1993), the activation by auxin of plant MAP kinase homologues (Mizouchi et al. 1994), the activation of phospholipase A2 by auxin (Gunther and Andre 1993), and increases in the expression of calmodulin (Jena et al. 1989) have been reported in plant cells. Recently it has been demonstrated that endogenous levels of cAMP show at least two sharp and transient accumulations during cell cycle progression of synchronized BY-2 cells (Ehsan et al. 1998). These reports may point to cAMP as being a possible player in the auxin transduction pathway regulating the progression through the cell cycle in higher plants.

In early 70s, some reports postulated cAMP to act as second messenger in the signal transduction pathway of auxin action (Salomon and Mascarenhas 1971, Srivastava et al. 1974, Kamiska and Masuda 1970). However, to our knowledge direct proof for the involvement of cAMP in the pathway of auxin

*A part of Ph.D. thesis of the first author. [1]Laboratorium voor Plantenbiochemie en-fysiologie, Department of Biology, Universiteit Antwerpen (UIA), Belgium. e-mail: hehsan@unh.edu

during the cell division cycle has never been reported. This paper reports the results of our experiments on the possibility of cAMP acting downstream auxin perception in the control of cell cycle progression of synchronized suspension cultures of tobacco BY-2 cells. This experimental system was chosen because of the high growth rate, homogeneity and synchronization potential of tobacco BY-2 cells (Nagata et al. 1992).

Materials and Methods

Culture maintenance, synchronisation and sampling: Tobacco BY-2 cell cultures were maintained according to the method described by Nagata et al. (1992). Releasing stationary cells into fresh media (20 - 200 ml) containing 5 µg/ml aphidicolin (Sigma) induced cell synchronization. Upon 24 h of aphidicolin treatment, early-S arrested cells were washed with 4 L of media and released into 200 ml of fresh media without 2,4-D. The suspension culture was split into four 50 ml portions to which various chemicals were added according to the experimental design. For auxin starvation experiments, seven days old stationary cells were released into fresh media (5 - 100 ml) containing no 2,4-D. After three days of starvation, cultures were washed with 2L of media and released into 100 ml of fresh media. This culture was then split either into four 25 ml or into two 50 ml portions to which various chemicals were added again depending on the kind of the experiment.

Mitotic index: One ml samples for mitotic index were collected at the time indicated. Upon sedimentation on ice and removal of the media, cells were fixed by adding 1 ml of ethanol/acetic acid (3 : 1 v/v) and stored at 4°C. The fixed cells were stained with 5µM DAPI (4´, 6-diamidino-2-phenylindole) and analyzed using a fluorescent microscope (Leitz, Germany) by counting the number of nuclei (300 cells) in late prophase to telophase.

Results and Discussion

In an initial experiment we applied 2,4-D (1 µM) or 1-NAA (10 µM), both with and without the anti auxin 2-NAA (100 µM), to A released cells (Fig. 1A). Regardless which auxin (2,4-D or 1-NAA) was used, the cells reached about 30% of synchronized mitosis 6 h after A release. Addition of 100 µM 2-NAA completely blocked the progression of the cell cycle. In order to investigate whether this effect of 2-NAA resulted from a competitive inhibition at the level of auxin perception, increasing amounts of the active auxin 1-NAA were added to the culture media containing 10 µM 2-NAA (Fig. 1b). The presence of 10 µM 2-NAA inhibited drastically cell cycle progression of synchronized BY-2 cells.

Addition of 1-NAA, even in a ten-fold higher concentration did not restore mitosis. A concentration of 5.5 μM 2-NAA induced only a partial reduction of the mitotic index, 22% compared to 35% in the control experiments (Fig. 1c). Again adding an equal amount of 1-NAA or the membrane permeable db-cAMP did not affect the inhibitory effect of this low 2-NAA concentration.

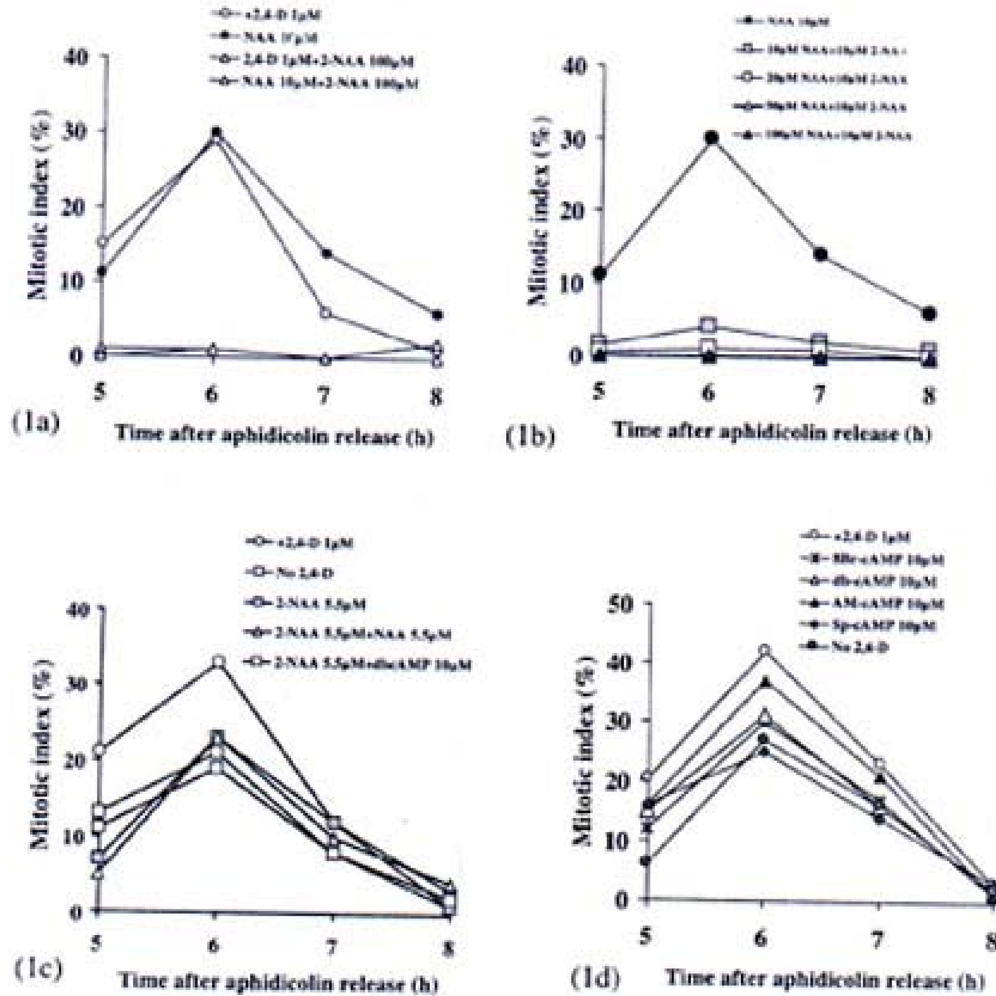

Fig. 1. Effect of auxins (2,4-D and 1-NAA) and various cAMP analogues (db-cAMP, 8Br-cAMP and Sp-cAMP) on mitotic division in aphidicolin released tobacco BY2-cells. (a) Induction/inhibition of mitotic division by 2,4-D, 1-NAA and 2-NAA. (b) Effect of increasing concentrations of NAA on the inhibitory effect of 10 μM of 2-NAA. (c) Effect of NAA and db-cAMP on a partially inhibiting concentration (5.5 μM) of 2-NAA. (d) Effect of c-AMP analogues (8Br-cAMP, db-cAMP and Sp-cAMP) and 10μM of AM-cAMP (an activator of cAMP dependent protein kinase) on mitosis in absence of 2,4-D.

These observations show that 2-NAA is either and most probably a rather unspecific inhibitor or that it acts downstream of auxin recognition. It certainly cannot be used as an anti-auxin with the purpose to identify compounds e.g. cAMP acting downstream of auxin perception in the cell cycle progression.

Omitting 2,4-D from the culture medium and replacing it by various membrane permeable cAMP analogues was another approach used to examine whether cAMP plays a role as an intermediate in the auxin signalling pathway leading to cell division. When immediately after A treatment, cells are washed and cultured in absence of 2,4-D a significant drop of the mitotic index (25% compared to 43% for the 2,4-D control) was observed (Fig. 1d). Cells that still proceeded through the cell cycle did so with the same speed as those

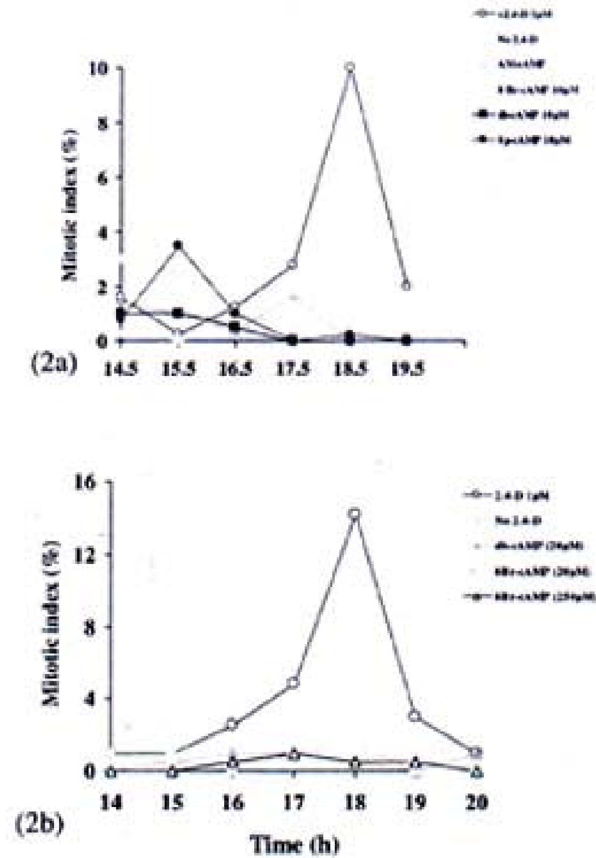

Fig. 2. Effect of 2,4-D (1µM) on reinitiating mitosis in auxin starved (72 h) tobacco BY2-cells. (a) Effect of 2,4-D (1 µM), various cAMP analogues (10 µM, 8-Br-cAMP, db-cAMP, Sp-cAMP) and 10 µM of AM-cAMP (an activator of cAMP dependent protein kinase). (b) Effect of higher concentrations of cAMP analogues.

growing in presence of 2,4-D. These observations can be explained by an incomplete removal of the 2,4-D, which results in a decreased mitotic index but did not alter the cell cycle speed. Adding 10µM of membrane permeable cAMP analogues (db-cAMP, Sp-cAMP, 8Br-cAMP) to the 2,4-D deprived cells did not significantly alter their mitotic index. The small differences observed could be related to a varying amount of residual 2,4-D. In presence 10 µM of AM-cAMP (an activator of cAMP dependent protein kinase), a slightly increased mitotic index was observed. However, it cannot be ruled out that this is also caused by an enhanced residual 2,4-D in these cells.

In order to avoid all possible effects due to residual 2,4-D, A treated cells were washed and kept in auxin free medium for 72 h. After auxin starvation, cells were extensively washed and split into several portions, to which either 2,4-D, or various cAMP analogues were added. (Fig. 2). Upon addition of 1 µM 2,4-D cells resumed cell cycle showing a mitotic index of 10 - 16% at about 18 h upon addition of 2,4-D. The results confirmed earlier observations (Ishida et al. 1993, Mizouchi et al. 1994) that even after three days of auxin starvation tobacco BY-2 cells remain sensitive to auxin perception and signaling leading to mitosis. In case cAMP acts downstream of auxin perception, adding membrane permeable but active cAMP analogues would be expected also to restore some mitosis in the auxin starved cells. However, the results shown in Fig. 2 give clear evidence that neither one of the added cAMP analogues (10 µM) nor the activator of cAMP-dependent protein kinase, AM-cAMP (10µM) was able to reinitiate cell cycle above the background levels as they were observed in absence of 2,4-D (Fig. 2 a). Even higher concentrations of db-cAMP or 8Br-cAMP (up to 250 µM) were ineffective in restoring mitosis (Fig. 2 b).

In conclusion, data presented in the paper show that cAMP plays an important role in the control of cell cycle progression of BY-2 cells (Ehsan et al. 1998), seemingly without influencing the signaling pathway downstream of auxin perception; at any rate it does not mimic auxin action in auxin depleted BY-2 cells.

Acknowledgements

The authors wish to thank Dr. Luc Roef and Erwin Witters for the fruitful discussions. This research was financed by a grant from the Belgian programme on interuniversity poles of attraction (Prime Minister's Office, Science programming, no. 15). H. V. O is a research Director at the Flemish Fund for Scientific Research, Belgium.

References

Ehsan H, Reichheld J-P, Roef L, Witters E, Lardon F, Van Bockstaele D, Van Montagu M, Inzé D and **Van Onckelen H** (1998) Effect of indomethacin on cell cycle dependent cyclic AMP fluxes in tobacco BY-2 cells. FEBS Letters **442** : 165-69.

Gunther FES and **Andre B** (1993) Stimulation of phospholipase A2 by auxin in microsomes from suspension-cultured soybean cells is receptor-mediated and influenced by nucleotides. Planta **191**: 515-523.

Ishida S, Takahashi Y and **Nagata T** (1993) Isolation of cDNA of an auxin-regulated gene encoding a G protein - subunit-like protein from tobacco BY-2 cells. Proc Natl. Acad. Sci, USA **90** : 11152-56.

Jena PK, Reddy ASN and **Poovaiah BW** (1989) Molecular cloning and sequencing of a cDNA for plant calmodulin: signal-induced changes in the expression of calmodulin. Proc Natl. Acad. Sci, USA **86** : 3644-48.

Mizouchi T, Gotoh Y, Nishida E, Yamaguchi-Shinozaki K, Hayashida N, Iwasaki T, Kamada H and **Shinozaki K** (1994) Characterization of two cDNAs that encode MAP kinase homologues in *Arabidopsis thaliana* and analysis of the possible role of auxin in activating such kinase activities in cultured cells. Plant J. **5**(1) : 111-22.

Kamiska S and **Masuda Y** (1970) Stimulation of auxin-induced cell expansion in plant tissue by 3´, 5´-adenosine monophosphate. Naturwissenschaften **57**(11) : 546.

Nagata T, Nemoto Y and **Hasezawa S** (1992) Tobacco BY-2 cell line as the “HeLa” cell in the cell biology of higher plants. Intl. Rev. Cytol. **132** : 1-30.

Salomon D and **Mascarenhas JP** (1971) Auxin-induced synthesis of cyclic 3',5'-adenosine monophosphate in *Avena* coleoptiles. Life Sciences [II]. **10**(15) : 879-85.

Srivastava A K, Azhar S and **Krishnamurti CR** (1974) Activation of adenyl cyclase in the early phase of germination. FEBS letters **47**(2) : 330-332.