Planta (2002) 216: 302–308
DOI 10.1007/s00425-002-0845-y

ORIGINAL ARTICLE

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# Auxin efflux carrier activity and auxin accumulation regulate cell division and polarity in tobacco cells

Received: 19 January 2002 / Accepted: 3 June 2002 / Published online: 14 August 2002


**Abstract** Division and growth of most types of in vitro-cultured plant cells require an external source of auxin. In such cultures, the ratio of external to internal auxin concentration is crucial for the regulation of the phases of the standard growth cycle. In this report the internal concentration of auxin in suspension-cultured cells of *Nicotiana tabacum* L., strain VBI-0, was manipulated either (i) by increasing 10-fold the normal concentration of 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid in the external medium; or (ii) by addition 1-N-naphthylphthalamic acid (NPA; an inhibitor of auxin efflux and of auxin efflux carrier traffic). Both treatments delayed the onset of cell division for 6–7 days without loss of cell viability. In both cases, cell division activity subsequently resumed coincident with a reduction in the ability of cells to accumulate [$^3$H]NAA from an external medium. Following renewed cell division, a significant proportion of the NPA-treated cells but not those grown at high auxin concentration, exhibited changes in the orientation of new cell divisions and loss of polarity. We conclude that cell division, but not cell elongation, is prevented when the internal auxin concentration rises above a critical threshold value and that the directed traffic of auxin efflux carriers to the plasma membrane may regulate the orientation of cell divisions.

**Keywords** Auxin carrier · 1-N-Naphthylphthalamic acid · *Nicotiana* (cell culture) · Phytotropin · Polar auxin transport

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**Abbreviations** 2,4-D: 2,4-dichlorophenoxyacetic acid · IAA: indole-3-acetic acid · NAA: 1-naphthaleneacetic acid · NPA: 1-N-naphthylphthalamic acid

## Introduction

The polar transport of auxins [indole-3-acetic acid (IAA) and related compounds] plays a key role in the regulation of auxin-dependent growth and developmental processes. Mediated auxin influx into individual cells is catalysed by specific auxin-anion uptake carriers, whilst efflux is catalysed by a different auxin-anion efflux carrier system (Rubery and Sheldrake 1974; Raven 1975; reviewed by Goldsmith 1977). Biochemical, physiological and molecular evidence indicates that the polarity of auxin transport through cells and tissues results from the polarised distribution of auxin efflux carriers in the plasma membrane (reviewed by Bennett et al. 1998; Morris 2000). Available evidence indicates that in contrast to the auxin uptake carrier, the efflux carrier is a much more complex system consisting of the transport catalyst itself and one or more associated regulatory proteins (Morris et al. 1991; Muday 2000). One of these regulatory proteins is believed to be a specific binding protein for phytotropins [1-N-naphthylphthalamic acid (NPA) and related compounds], which may be associated with the actin cytoskeleton (Muday 2000; Muday and DeLong 2001; Muday and Murphy 2002). Phytotropins are potent non-competitive inhibitors of auxin efflux (and, consequently, stimulators of net auxin accumulation) and of polar auxin transport (Rubery 1990).

Cell-suspension cultures provide good model systems in which the effects of growth substances on cell division and growth can be studied directly. In such cultures the rate and intensity of cell division are regulated, among other things, by changes in the external and internal concentrations of two essential phytohormones, auxins and cytokinins. Manipulation of the levels of these hormones in tobacco cell cultures (and, consequently,

the ratio of their momentary concentrations in the cultivation medium) may result in a change of developmental programme from cell elongation to cell division (Hasezawa and Syono 1983; Stickens et al. 1996).

Earlier we described the relationships between internal auxin (IAA) level and auxin-binding activity in relation to cell division intensity in the auxin-dependent and cytokinin-autonomous tobacco cell strain VBI-0 (Zažímalová et al. 1995). During the exponential (rapid cell division) phase, reduction in the external auxin concentration resulted in a substantial decrease of cell division activity but increased the internal level of free IAA in cells and the activity of a membrane-bound auxin-binding site. The strong inhibition of auxin efflux by NPA greatly increases auxin accumulation in NPA-treated tissues (Morris and Robinson 1998). Similarly, increases in the external concentration of auxin also strongly promote total auxin accumulation (Johnson and Morris 1989). To investigate the effects of raised internal auxin levels on cell division activity and cell phenotype in VBI-0 suspension cultures, we have examined the response of VBI-0 cells to the inclusion of NPA in the culture medium, or to increases in the concentration of auxin supplied in the medium. Here we report that whilst both treatments result in a temporary and reversible inhibition of cell division activity, treatment with NPA, but not with high external auxin concentrations, causes partial loss of cell polarity and disturbed orientation of cell division when division activity resumes.

## Materials and methods

### Plant material

The auxin-dependent and cytokinin-autonomous VBI-0 tobacco cell strain derived from the stem pith of *Nicotiana tabacum* L., cv. Virginia Bright Italia (Opatrný and Opatrná 1976) was cultivated in standard Heller liquid medium (Heller 1953) supplemented with synthetic auxins 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D; 5.4 μM, and 4.5 μM, respectively). Cells were sub-cultured every 2 weeks (inoculation density approx. $5\times10^4$ cells $ml^{-1}$) and cultivated at 25 °C in darkness on an orbital shaker (INR-200; Sanyo–Gallenkamp, UK) at 120 rpm (diameter 32 mm).

### Determination of cell population density and viability test

Cell densities were determined by counting cells in at least 10 aliquots of each culture sample using a Fuchs-Rosenthal haemocytometer slide. Cell viability was assessed by the Trypan Blue dye exclusion test (Phillips 1973). 0.5 ml of 0.4% Trypan Blue solution (Sigma) was mixed with 0.5 ml of cell suspension and cells were examined microscopically within 5 min. The percentage of viable (unstained) cells was determined from at least 10 optical fields on each of 3 separate slides.

### Microscopic examination of cells

Cells were examined with an Olympus Provis AX-70 microscope equipped with Nomarski DIC optics and an automatic photomicrography system. Images were stored on a computer for electronic processing. The frequency of longitudinal and oblique cell divisions in control (no NPA) and NPA-treated cells (expressed as a percentage of the dividing cells) was determined by microscopic examination of 5 separate samples per treatment (500 cells were assessed in each sample).

### NPA treatments

NPA was synthesised at the Institute of Experimental Botany, Prague, by the method of Meyer and Wolfsleben (1911), and its quality and purity were checked by melting-point determination, infrared spectroscopy, thin-layer chromatography and HPLC.

In experiments in which NPA was included in the cultivation medium, it was added as a 5 mM filter-sterilised stock solution in 96% ethanol directly to the cultivation medium at the start of the sub-culture interval to a final concentration of 10, 50 and 100 μM. An equivalent volume of 96% ethanol was added to control cultures. In other experiments, NPA was included only during the accumulation assays and was added to the uptake buffer to give the required final concentration. An equivalent volume of 96% ethanol was added to control assays.

### Auxin-accumulation assay

The accumulation by the cells of [$^3$H]NAA (specific radioactivity 935 GBq $mmol^{-1}$; synthesised at the Isotope Laboratory, Institute of Experimental Botany, Prague, Czech Republic) was measured in 0.5-ml aliquots of cell suspension (cell density ca. $2\times10^5$ cells $ml^{-1}$) by a method modified from the protocol of Delbarre et al. (1996). Briefly, each cell suspension was filtered, re-suspended in uptake buffer (20 mM Mes, 40 mM sucrose, 0.5 mM $CaSO_4$, pH adjusted to 5.7 by KOH) and equilibrated for 45 min with continuous orbital shaking. Equilibrated cells were collected by filtration, re-suspended in fresh uptake buffer and incubated on the orbital shaker for 1.5 h in darkness at 25 °C. [$^3$H]NAA was added to the cell suspension to give a final concentration of 2 nM. At timed intervals (6 or 20 min according to experiment), 0.5-ml aliquots of suspension were withdrawn and accumulation of label was terminated by rapid filtration under reduced pressure on 22-mm-diameter cellulose filters. The cell cakes and filters were transferred to scintillation vials, extracted in 0.5 ml ethanol for 30 min and radioactivity was determined by liquid scintillation counting. Counts were corrected for surface radioactivity by subtracting counts obtained for aliquots of cells withdrawn immediately after the addition of [$^3$H]NAA. Treatments were replicated at least three times and averaged values (± standard errors), expressed as pmol NAA accumulated per $10^6$ cells, are shown below.

## Results

### Cell division in VBI-0

VBI-0 cells inoculated into media containing 50 μM NPA showed a remarkably different behaviour from control cells (Figs. 1, 2a). NPA treatment resulted in an almost complete cessation of cell division for the first 6 days of cultivation, accompanied by a stimulation of cell elongation (cf. Fig. 1b and Fig. 1e). The cessation of cell division was only temporary, however, and after several days (usually 6–9) the cells started to divide again (Fig. 1f, g). As revealed by the Trypan Blue test, cell viability was not affected by NPA treatment and was between 79 and 89% for control cells and between 81 and 93% for NPA-treated cells. Standard deviations of

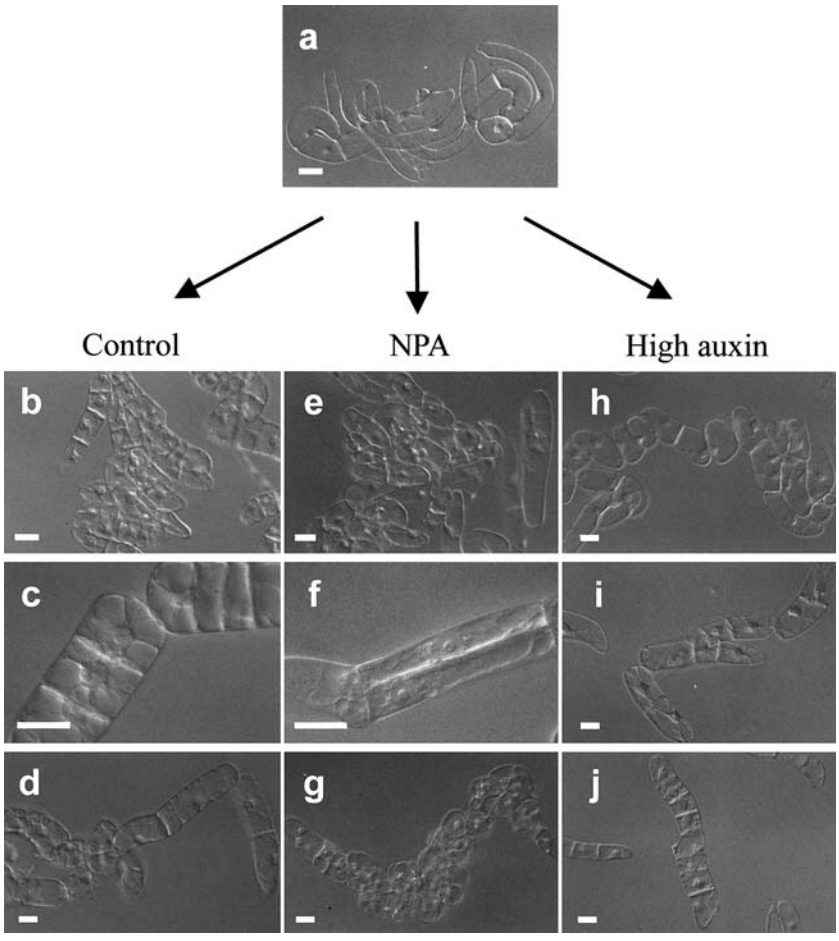


**Fig. 1a–j** Effects of NPA and of high external auxin concentration on cell division activity and phenotypic characters of suspension-cultured cells of tobacco (*Nicotiana tabacum*, line VBI-0). **a** Two-week-old inoculum consisting of individual elongated cells. **b–d** Cells grown in control medium (5.4 μM NAA plus 4.5 μM 2,4-D; no NPA) for 3, 6 and 9 days, respectively. **e–g** Cells grown for 3, 6 and 9 days, respectively, in control medium (as above) supplemented with NPA (50 μM). **e** Inhibition of cell division is apparent after 3 days. **f** Axial (longitudinal) divisions in 6-day-old cells. **g** Renewal of cell division activity and formation of non-polarised cell clusters 9 days after start of NPA treatment. **h–j** Cells grown for 3, 6 and 9 days, respectively, in the presence of high external auxin concentrations (54 μM NAA plus 45 μM 2,4-D; no NPA). **h** 3-day-old cells in which cell division has been inhibited. **i, j** Cells grown for 6 and 9 days, respectively, at high external auxin concentration showing renewed cell division activity and the development of polarised, one-cell wide filaments as a result of regular transverse divisions. Bars = 50 μm

cell viability determinations were between 4 and 6% of the means for both control and treated cells.

Similar effects on cell division and cell elongation were observed in cells cultured in media containing 10 times the normal concentrations of NAA and 2,4-D (Figs. 1h–j, 2b). Cell division activity was suppressed by the high auxin concentration but, as with NPA, the rate of cell division began to increase again after about 6 days (Fig. 1i, j). Similar to the NPA treatment, cell growth continued during the period of suppressed division activity (Fig. 1h). Like treatment with NPA, raising the external auxin concentration 10-fold did not affect cell viability (data not shown).

## Accumulation of [$^3$H]NAA

To check whether high NPA concentrations might have damaging effects on cells, the effects of [$^3$H]NAA accumulation by VBI-0 cells were tested by exposing cells to a range of NPA concentrations between 1 and 100 μM (Fig. 3). Inhibition of [$^3$H]NAA efflux (i.e. stimulation of NAA accumulation) by NPA in VBI-0 cells was saturated between 1 and 30 μM. Since 50 μM NPA had a maximal and consistent effect on auxin accumulation with no obvious signs of cell damage, this concentration was chosen as a standard for the present study.

To confirm that NPA treatment caused an increase in auxin accumulation, we measured net accumulation of [$^3$H]NAA (2.0 nM) by cells cultured in standard medium and in medium supplemented with 50 μM NPA from the start of the sub-culture period. Accumulation was measured over a 6-min uptake period. In these experiments, NPA was not included in the uptake buffer. Previous results have demonstrated that no substantial metabolism of [$^3$H]NAA occurs over this time interval (Delbarre et al. 1996). As expected, inclusion of NPA in the cultivation medium substantially increased the abil-

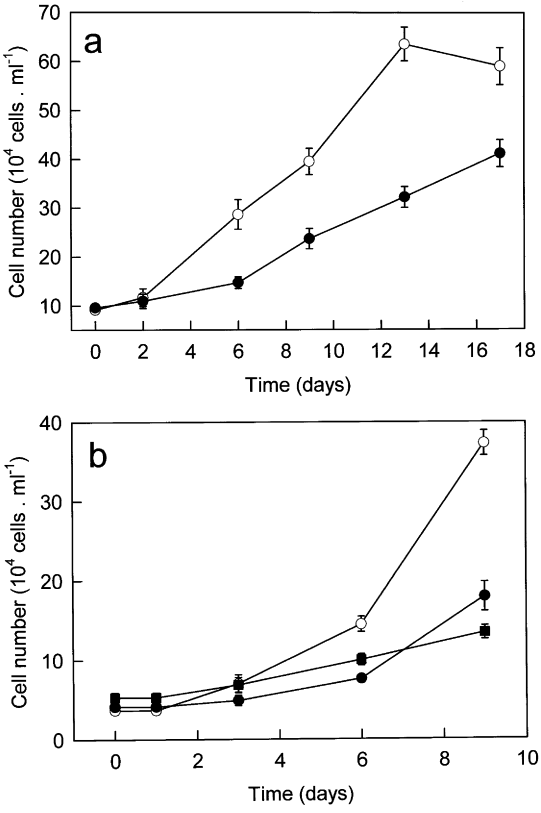


**Fig. 2a, b** Changes with time in cell densities during the suspension culture of tobacco VBI-0 cells. **a** Delay in the onset of the exponential cell division phase in cells cultured in the presence (*filled circles*) or absence (*open circles*) of NPA (50 μM). **b** Inhibition of cell division in VBI-0 cells grown in the presence of high (*filled circles*; 54 μM NAA plus 45 μM 2,4-D) or normal (*open circles*; 5.4 μM NAA plus 4.5 μM 2,4-D) external auxin concentrations. The effects of NPA (50 μM), also included in this experiment, are shown for comparison (*filled squares*). Values represent arithmetic means ± SE ($n=10$)

ity of the cells to accumulate [$^3$H]NAA (Table 1). However, both accumulation by control cells (no NPA) and the magnitude of the response to NPA changed dramatically with progress of the culture cycle (Fig. 4, Table 1). Maximum levels of accumulation and greatest responses to NPA both occurred in the period 2–5 days after the initiation of the cultures, corresponding to the start of the exponential phase in control cells and the time when NPA had its greatest effect on cell division activity (Fig. 4, Table 1).

### Phenotypic effects of NPA

Despite their similar effects in delaying the exponential phase of cell division, the responses to high external auxin concentrations and to NPA treatment differed in an important respect following the resumption of cell division. Following renewed cell division activity, cells exposed to high auxin concentrations gave rise to a normal polar filamentous phenotype by repeated

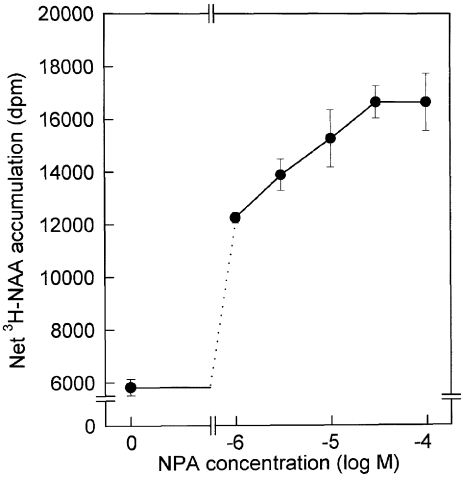


**Fig. 3** Effect of concentration of NPA on the net accumulation of [$^3$H]NAA (2 nM) by exponential (6-day-old) suspension-cultured tobacco VBI-0 cells (20 min uptake period). Values represent arithmetic means ± SE ($n=8$)

transverse cell divisions (cf. Fig. 1c, d and Fig. 1i, j). In a substantial proportion of the cells treated with NPA (50 μM), however, the orientation of cell division following recovery was frequently oblique or even longitudinal (Fig. 1f). This sometimes resulted in the formation of cell clumps rather than linear filaments (Fig. 1g).

We have compared the occurrence of longitudinal or oblique cell divisions at a range of NPA concentrations between 0 and 100 μM (Fig. 5). There were no qualitative differences in the appearance of cell clumps arising from abnormal divisions at 10 μM NPA (Fig. 5b), 50 μM NPA (Fig. 1g), and 100 μM NPA. However, the proportion of abnormally aligned cell division axes was lower at 10 μM NPA and increased with increasing NPA concentration (Fig. 5c).

The percentage of occurrence of abnormal cell divisions varied depending on the inoculum density: the proportion of longitudinal or oblique cell divisions was higher at lower inoculum densities ($1\times10^4$–$2\times10^4$ cells $ml^{-1}$). There was no effect on the phenotype when NPA was applied at the end of the exponential growth phase, i.e. the late NPA application did not influence either cell elongation or the establishment of cell polarity (data not shown).

## Discussion

The tobacco cell line VBI-0 possesses several unique characteristics which, together with its good phenotypic stability, makes it an excellent model system for cytological and biochemical studies. These characteristics include a high spontaneous friability (which reduces the tendency of the cells to form clumps in suspension cultures), a filamentous phenotype, polar growth of cells and filaments, and a well-defined temporal separation of cell division and cell elongation phases (Zažímalová et al.

**Table 1** Effects of cultivation of VBI-0 cells in the presence of 50 μM NPA on the changes with time in net [$^3$H]NAA accumulation (6 min uptake period in sub-samples of cells collected on the days shown) and on cell densities during the early stages of growth of VBI-0 suspension cultures. Values are arithmetic means ± SE ($n = 10$ for cell densities and $n = 3$ for auxin accumulation). *n.d.* Not detected

| Day | Net [$^3$H]NAA accumulation (pmol $10^{-6}$ cells) | | | Cell number ($10^4$ cells ml$^{-1}$) | | |
|---|---|---|---|---|---|---|
| | Control | 50 μM NPA | Response to NPA (% of control) | Control | 50 μM NPA | Response to NPA (% of control) |
| 0 | 0.16 ± 0.02 | 0.16 ± 0.02 | 0 | 5.18 ± 0.50 | 5.18 ± 0.50 | 0 |
| 3 | 5.10 ± 0.32 | 12.82 ± 0.26 | 151.4 | 8.17 ± 0.65 | 5.52 ± 0.46 | 67.6 |
| 5 | 0.59 ± 0.26 | 4.89 ± 0.26 | 728.8 | 12.27 ± 1.40 | 7.71 ± 0.53 | 62.8 |
| 9 | n.d. | n.d. | – | 37.51 ± 1.90 | 12.12 ± 0.56 | 32.3 |

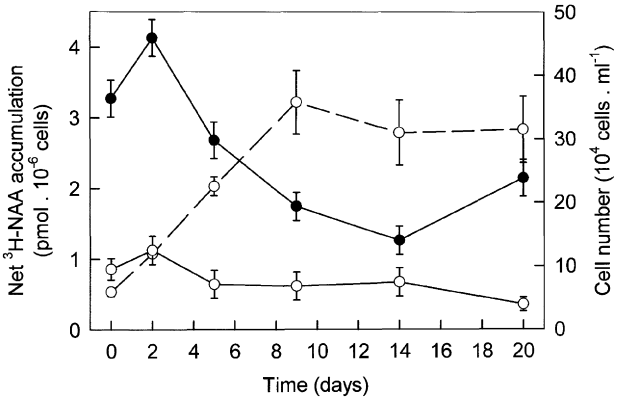


**Fig. 4** Changes in rate of accumulation of [$^3$H]NAA (2 nM; 6 min uptake period) during the growth cycle of suspension-cultured tobacco VBI-0 cells, measured in the presence (*filled circles*) or absence (*open circles*) of NPA (50 μM; *unbroken line*). Changes with time in the cell density of control cultures (*open circles*; *broken line*) are shown. Values represent arithmetic means ± SE ($n = 10$ for cell densities and $n = 3$ for auxin accumulation)

1995, 1996; Petrášek et al. 1998). The strain is auxin-dependent and the progress of its growth cycle, including duration of the lag phase, rate of cell division, and duration of the exponential phase, is closely correlated with the particular concentration of auxin in the cultivation medium. The external auxin concentration also has been shown to regulate the concentration of native auxin (IAA) inside the cells (Zažímalová et al. 1995).

In the present study we investigated the effects on cell division and on cell elongation in suspension-cultured VBI-0 cells of manipulating internal auxin concentration by two contrasting methods: firstly, by changing the auxin concentration in the medium in which the cells were cultured (Zažímalová et al. 1995); and secondly, by inhibiting auxin efflux (thereby stimulating net auxin accumulation) by exposing the cells to NPA, a compound which strongly inhibits auxin efflux carrier activity (Rubery 1990; see also Delbarre et al. 1996).

Taken together, the results presented here indicate that there is a critical threshold concentration of internal auxin above which cell division cannot proceed but above which cell elongation can continue or may even be promoted. Thus factors regulating internal auxin concentration, including auxin influx from and efflux to the external medium, may differentially regulate cell elongation and cell division. Furthermore, the data presented in Fig. 4 and Table 1 demonstrate that substantial temporal changes in auxin efflux carrier activity occur during the normal course of the growth cycle in cultured VBI-0 cells. Thus the onset and duration of the exponential and stationary phases of growth in vitro normally may be controlled by the regulation of the activity of auxin carrier systems.

Of particular significance was the observation that treatment of the cells with NPA caused a marked change in their phenotypic behaviour after competency to divide recovered. On the other hand, cells cultured at high external auxin concentrations recovered to produce normal, linear cell filaments. The reasons for these different responses may reside in the mechanism by which NPA inhibits auxin efflux. It is now well established that the half-life of auxin carriers in the plasma membrane is very short (Delbarre et al. 1998; Morris and Robinson 1998; Robinson et al. 1999; Steinman et al. 1999) and that they may cycle rapidly between the plasma membrane and an as yet unidentified endomembrane compartment (Robinson et al. 1999; Geldner et al. 2001, and references therein). Recently it has been reported that the inhibitor of polar auxin transport 2,3,5-triiodobenzoic acid (TIBA; and possibly also NPA – although no details of the latter were presented) prevents the traffic of PIN1 (a putative auxin efflux catalyst) and other rapidly cycled proteins to and from the plasma membrane in *Arabidopsis* root cells (Geldner et al. 2001). Consequently, the inhibition of auxin efflux by auxin transport inhibitors conceivably might be caused by net loss of functional efflux carriers from the plasma membrane, or by other non-specific effects of these compounds on protein traffic, rather than by inhibition of the transport catalytic activity of plasma membrane-located efflux carriers per se. This also might explain why NPA and brefeldin A (an inhibitor of vesicle traffic) mimic each other in their effects on auxin transport (Robinson et al. 1999; Geldner et al. 2001). However, it should be noted that despite the potential importance of the claimed role for

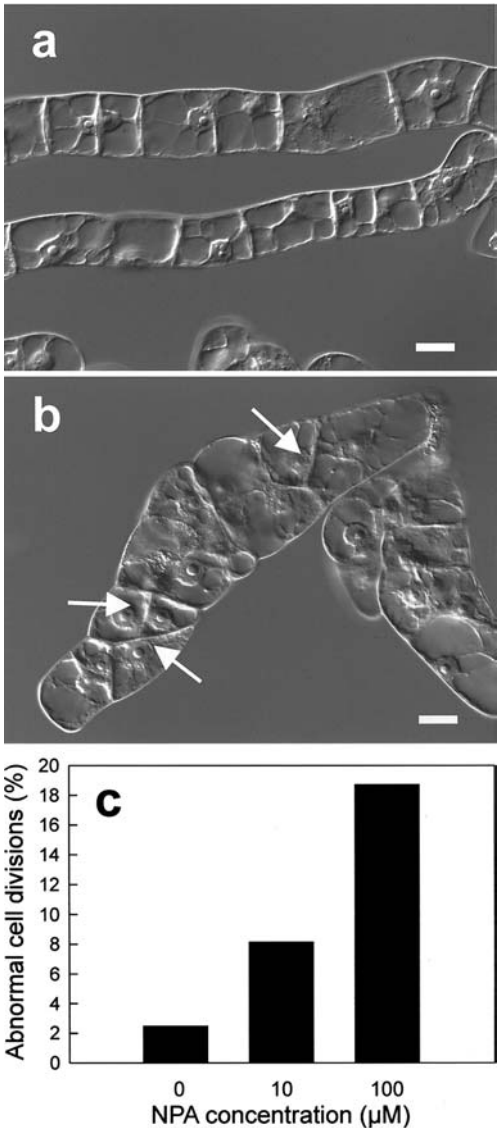


**Fig. 5a–c** Effects of NPA (10 μM) on phenotype of suspension-cultured tobacco VBI-0 cells. **a** Cells grown in control medium, day 9. **b** Cells grown in control medium supplemented with NPA (10 μM), day 9. Note abnormal cell division planes (*arrows*). Bars in **a** and **b** = 50 μm. **c** Effect of concentration of NPA on the occurrence of abnormal cell divisions at day 9. Standard deviations of the means were less than 10 and 12% of the mean values for control and NPA-treated cells, respectively ($n = 5$; 500 cells per sample)

NPA in inhibiting protein traffic, no experimental data on NPA were shown in the paper by Geldner et al. (2001). Furthermore, the concentration of NPA (200 μM) reported by these authors to be necessary to cause a general perturbation of protein trafficking to and from the plasma membrane is some 2 orders of magnitude greater than that commonly reported to be effective in inhibiting auxin efflux. In the VBI-0 cell line used in our experiments as little as $10^{-6}$ M NPA caused a substantial (more than 2-fold) increase in the net accumulation of [$^3$H]NAA (Fig. 3). Indeed, given these very high concentrations of NPA, the possibility cannot be excluded that the reported effects of NPA on protein traffic (Geldner et al. 2001) are side effects of high NPA concentrations that are unrelated to the effects of NPA on auxin efflux.

We reported elsewhere (Černá et al. 2002) that whilst treatment with either brefeldin A (20 μM) or NPA (50 μM) results in a very rapid and substantial increase in the rate of [$^3$H]NAA accumulation by suspension-cultured BY-2 tobacco cells, consistent with disruption of efflux carrier activity at the plasma membrane, only brefeldin A affected the arrangement of the actin cytoskeleton, which is believed to be involved in auxin efflux carrier traffic to the plasma membrane (reviewed by Muday and Murphy 2002). Thus, in contrast to the results reported by Geldner et al. (2001), in our hands the concentrations of NPA that induce a marked increase in NAA accumulation by tobacco cells appear not to disrupt actin-dependent efflux carrier protein traffic.

As pointed out by Muday and DeLong (2001), the mechanism of action of NPA on membrane protein cycling remains completely unknown. Thus, the possibility cannot completely be excluded that the changes in cell polarity following NPA treatment may result from more general, but as yet unsubstantiated, effects of NPA on the targeting of rapidly turned-over proteins. However, it is more likely that the observed abnormalities in the planes of cell division and developmental polarity following the NPA treatment resulted from misdirected targeting of auxin efflux carriers themselves to the plasma membrane during the recovery period. If this is true, then it follows that the site-directed traffic of auxin carriers to the plasma membrane may play a crucial role in the regulation of both cell division activity and the establishment of cellular polarity.

**Acknowledgements** The work was supported by the Grant Agency of the Czech Republic (project No. 206/98/1510), by the EU INCO Copernicus (project No. ERBIC15 CT98 0118) to E.Z. and by grants to D.A.M. from the UK Royal Society and the Academy of Sciences of the Czech Republic under the European Science Exchange Scheme. The authors thank Andrea Hourová for her excellent technical assistance.

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