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Vinblastine and nocodazole inhibit basal and thyrotropin-releasing hormone-stimulated prolactin secretion in GH_3 cells

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To investigate the efficacy of vinblastine as a possible therapeutic agent in prolactinomas, we have examined the effects of vinblastine on GH_3 cell function. The effects of vinblastine were compared to another anti-microtubule drug, nocodazole. At 24 h, prolactin (PRL) secretion was 737 ± 63 ng/ml in control cells. In cells treated with 0.1, 1 and 10 μM vinblastine, PRL secretion was reduced to 370 ± 68, 344 ± 25 and 125 ± 31 ng/ml, respectively. In cells treated with 10 μM nocodazole for 24 h, PRL secretion was reduced to 200 ± 30 ng/ml. After a 24 h incubation with the drugs, cells were washed with drug-free medium and challenged with 100 nM TRH for 10 min. TRH-stimulated PRL secretion was 35 ± 7 ng/ml in control cells, 14 ± 0.5 ng/ml in vinblastine-treated cells and 8.8 ± 0.1 ng/ml in nocodazole-treated cells. [^{3}H]TRH binding to GH_3 cell membrane was inhibited by about 15% by vinblastine and nocodazole. In vinblastine and nocodazole treated cells, polymerized tubulin levels decreased by 46 and 55%, respectively. These observations that vinblastine suppresses PRL secretion by GH_3 cells suggest that this drug might be useful as a therapeutic agent for prolactinomas.

Keywords: GH_3 cells; tubulin; vinblastine; nocodazole; prolactin

Introduction

Clinically, *Vinca* alkaloids (vinblastine or vincristine) are perhaps the most widely used chemotherapeutic agents (Rowinsky & Donehower, 1991). In mammals, *Vinca* alkaloids inhibit tumor growth by mitotic arrest. These alkaloids block mitosis by their ability to depolymerize microtubules. At low concentrations (1 to 2 μM) vinblastine causes splaying and peeling of protofilaments in the cell resulting in the depolymerization of microtubules. At higher concentrations (> 10 μM), vinblastine binding to tubulin results in tubulin self-association leading to the formation of tubulin paracrystals in cells (Hamel, 1990). Tubulin, the monomeric unit of microtubules, has a molecular weight of about 100 kDa and is composed of two dissimilar α and β subunits. In addition to serving as a component of cytoarchitecture, microtubules, because of their ability to depolymerize and repolymerize, are important to many cellular functions, such as, the intracellular movement of prolactin (PRL) secretory granules and their secretion (Ravindra & Grosvenor, 1990).

Although *Vinca* alkaloids are widely used in the therapy of many types of tumors, the effect of these compounds in diminishing prolactin-secreting pituitary adenomas is largely unexplored. The present study is an attempt to investigate the efficacy of vinblastine as a possible therapeutic agent in prolactinomas. We have chosen to study the effect of vinblastine on PRL secretion by the rat pituitary tumor-derived GH_3 cells. Moreover, in an attempt to compare the effect of vinblastine to another anti-microtubule agent, the effect of a synthetic compound, nocodazole on PRL secretion by GH_3 cells has been investigated. GH_3 cells, although tumor cells, retain many of the differentiated features of anterior pituitary cells and are an established model to study PRL secretion (Tashjian, 1979).

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Results

Effect of vinblastine and nocodazole on cellular protein levels

Drugs were added to 80% confluent GH_3 cell cultures. After a 24 h incubation with the drugs, protein concentrations in the cell homogenates were estimated. Protein concentration in control cells was 2.3 ± 0.01 mg/ml. In cells treated with 10 μM vinblastine or nocodazole the protein concentration was 1.6 ± 0.1 and 1.9 ± 0.04 mg/ml, respectively (significantly different fron the control value, $P<0.05$; Figure 1).

Effect of vinblastine and nocodazole on PRL secretion

A time-course study revealed that vinblastine (1 μM) and nocodazole (10 μM) inhibited PRL secretion by GH_3 cells at 16 and 24 h. At 16 h PRL secretion in control, vinblastine- and nocodazole-treated cells was 732 ± 57 388 ± 48 and 386 ± 15 ng/ml, respectively (significantly different from the control value, $P<0.05$; Figure 2). At 24 h PRL secretion in control, vinblastine- and nocodazole-treated cells was 832 ± 30 220 ± 15, and 180 ± 3.5 ng/ml, respectively (significantly different from the control value, $P<0.05$; Figure 2).

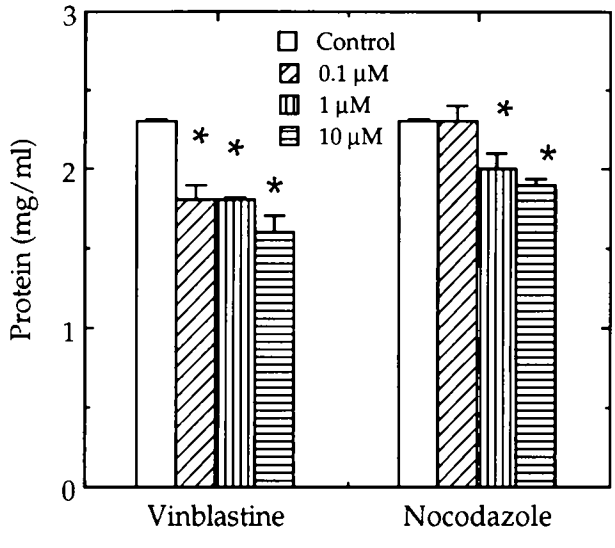

Figure 1 Influence of drugs on cellular protein concentration. Drugs were added to 80% confluent GH_3 cell cultures. After a 24 h incubation with the drugs, cells were harvested and protein concentration in the cell homogenate was estimated. Each point represents the mean ± SEM of three determinations. Asterisk (*) indicates value significantly different from the control value ($P<0.05$)

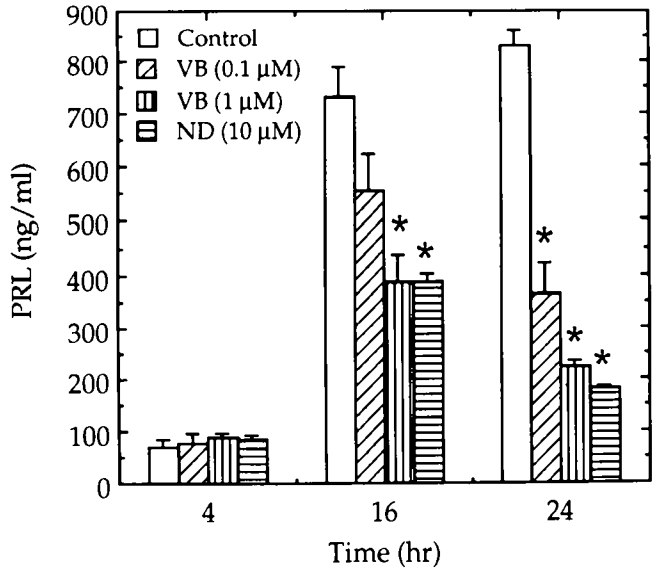

Figure 2 Time course effect of drugs on PRL secretion by GH_3 cells. GH_3 cells were incubated with vinblastine (VB) or nocodazole (ND) in serum-free medium for the indicated periods of time, and PRL in medium was determined. Each point represents the mean ± SEM of three determinations. Asterisk (*) indicates value significantly different from the control value ($P<0.05$)

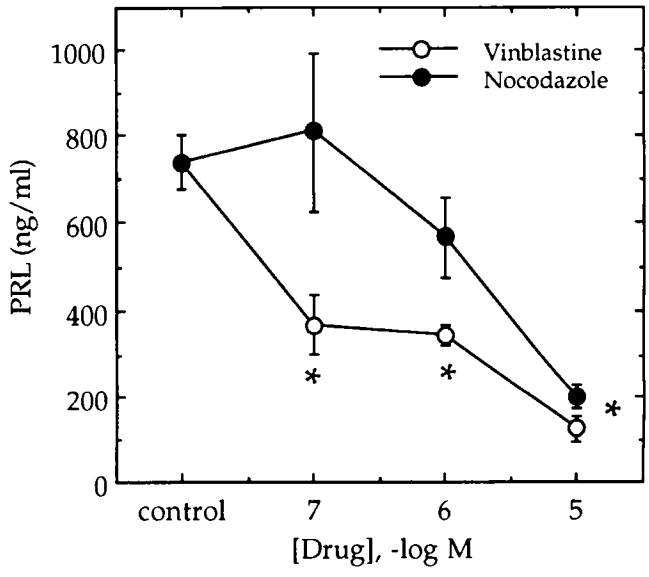

Figure 3 Effect of vinblastine and nocodazole on PRL secretion by GH_3 cells. GH_3 cells were incubated with various concentrations of vinblastine or nocodazole in serum-free medium for 24 h and PRL in medium was determined. Each point represents the mean ± SEM of three determinations. Asterisk (*) indicates value significantly different from the control value ($P<0.05$)

Incubation of GH_3 cells with vinblastine or nocodazole for 24 h resulted in an inhibition of PRL secretion. PRL secretion was 737 ± 63 ng/ml in control cells. In cells treated with 0.1, 1 and 10 μM vinblastine, PRL secretion was 370 ± 68 344 ± 125 and 125 ± 31 ng/ml, respectively (significantly different from the control value, $P<0.05$; Figure 3). In cells treated with 10 μM nocodazole, PRL secretion was 200 ± 30 ng/ml (significantly different from the control value, $P<0.05$; Figure 3).

Vinblastine (1 and 10 μM) and nocodazole (10 μM) suppressed TRH-stimulated PRL secretion by GH_3 cells. After a 24 h incubation with these concentrations of vinblastine or nocodazole, cells were washed with drug-free medium and challenged with 100 nM TRH for 10 min. It was determined to challenge the cells with TRH for 10 min because it was observed that 100 nM TRH stimulated the hormone secretion by 119, 190 and 200% at 1, 5 and 10 min, respectively (Ravindra *et al.*, unpublished observations). TRH-stimulated PRL secretion was 35 ± 7 ng/ml in control cells, 14 ± 0.5 ng/ml in vinblastine-treated cells, and 8.8 ± 0.1 ng/ml in nocodazole-treated cells (significantly different from the control value, $P<0.05$; Figure 4).

In order to determine whether the inhibitory effect of the drugs was reversible, cells were incubated for 24 h with the drugs, washed and incubated in drug-free medium. PRL secretion was determined at 24 and 48 h after drug withdrawal. Drug-treated cells did not recover the ability to secrete PRL secretion. At 24 h, cumulative PRL secretion was 1033 ± 155 ng/ml in control cells, 52 ± 10 ng/ml in nocodazole-treated cells and 206 ± 14 ng/ml in vinblastine-treated cells (significantly different from the control value, $P<0.05$; Figure 5). At 48 h, cumulative PRL secretion was 1419 ± 25 ng/ml in control cells, 36 ± 14 ng/ml in nocodazole-treated cells and 288 ± 24 ng/ml in vinblastine-treated cells (significantly different from the control value, $P<0.05$; Figure 5). At 48 h after drug withdrawal, TRH-stimulated PRL secretion was 21 ± 3.5 ng/ml in control cells, 4.4 ± 0.7 ng/ml in nocodazole-treated cells and 10 ± 1.4 ng/ml in vinblastine-treated cells (significantly different from the control value, $P<0.05$; Figure 6). These results indicated that the cells do not recover from the effects of the drugs after the removal of the drugs.

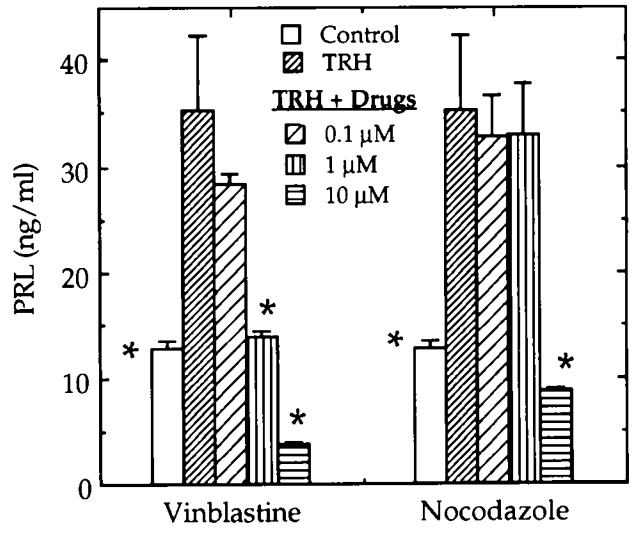

Figure 4 Effect of vinblastine and nocodazole on TRH-stimulated PRL secretion by GH_3 cells. After a 24 h incubation with various concentrations of the drugs, cells were washed with drug-free medium and challenged with 100 nM TRH for 10 min and PRL in medium was determined. Each point represents the mean ± SEM of three determinations. Asterisk (*) indicates value significantly different from the TRH value ($P<0.05$). One of two similar experiments is shown

Since the drugs caused a significant decrease in the amount of cellular protein concentration, it is reasonable to expect that PRL secretion profiles will change if expressed as ng/mg protein. The results presented in Figure 3 were expressed as ng/ml, and it was observed that 0.1, 1 and 10 μM vinblastine inhibited PRL secretion by 50, 53 and 83%, respectively; 10 μM nocodazole suppressed PRL secretion by 73%. When the data was expressed as ng/mg protein, there was no difference in the pattern of inhibition of hormone secretion by the drugs. PRL secretion was inhibited by 36, 40 and 76% with 0.1, 1 and 10 μM vinblastine; 10 μM nocodazole suppressed PRL secretion by 78%. Thus, the magnitude of change in

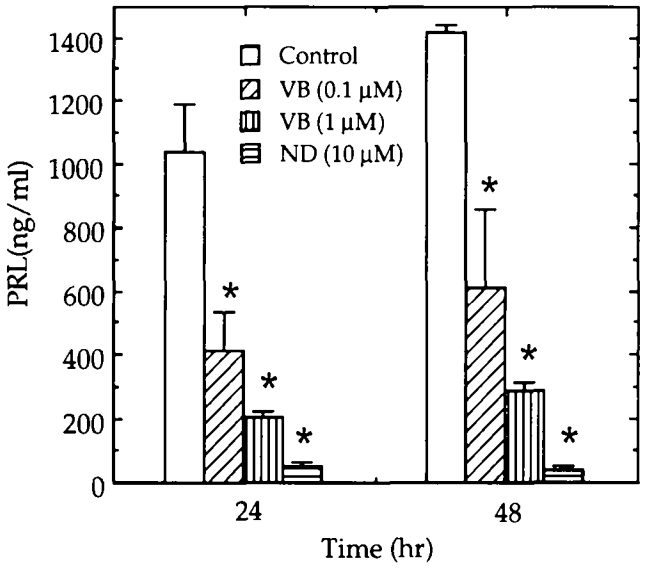

Figure 5 PRL secretion by GH_3 cells after drug removal. Cells were incubated for 24 h with vinblastine (VB) or nocodazole (ND), washed and incubated in drug-free medium and PRL secretion determined at 24 and 48 h after drug withdrawal. Each point represents the mean ± SEM of three determinations. Asterisk (*) indicates value significantly different from the control value ($P<0.05$)

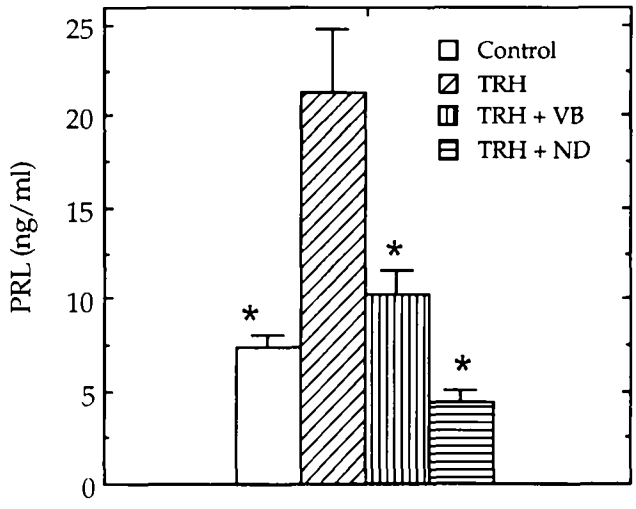

Figure 6 TRH-stimulated PRL secretion by GH_3 cells after drug removal. Cells were incubated for 24 h with vinblastine (VB, 1 μM) or nocodazole (ND, 10 μM), washed and incubated in drug-free medium. At 48 h after drug withdrawal, cells were challenged with 100 nM TRH for 10 min and PRL in medium was determined. Each point represents the mean ± SEM of three determinations. Asterisk (*) indicates value significantly different from the TRH value ($P<0.05$). One of two similar experiments is shown

PRL secretion is such that it did not make any difference whether the data was expressed as ng/ml or ng/mg protein. In addition, in both instances the drug-induced inhibition was statistically significant.

Effect of vinblastine and nocodazole on [^{3}H]TRH binding to GH_3 cell membranes

We examined the possibility that vinblastine and nocodazole suppressed TRH-stimulated PRL secretion by inhibiting the binding of the hormone to its receptor. [^{3}H]TRH binding to GH_3 cell membranes was inhibited by about 15% by vinblastine or nocodazole (significantly different from the control value, $P<0.05$; Figure 7).

Effect of vinblastine and nocodazole on soluble and polymerized tubulin levels

It was determined if the effects of the drugs on PRL secretion are accompanied by their ability to depolymerize microtubules in GH_3 cells. After incubating with the drugs for 24 h, GH_3 cells were homogenized and soluble and polymerized tubulin fractions were extracted from the homogenate as described in Materials and methods. The high-speed supernatant of the homogenate represents the soluble fraction; the resulting pellet was resuspended in the extraction buffer and the high-speed supernatant of this suspension represents the polymerized fraction. The protein concentration in the soluble tubulin fraction was 1.63, 1.54 and 1.52 mg/ml in control, nocodazole-, and vinblastine-treated samples, respectively. The protein concentration in the polymerized tubulin fraction was 1.15, 0.43 and 0.36 mg/ml in control, nocodazole-, and vinblastine-treated samples, respectively. At first glance, these values appear to be contradictory to those presented in Figure 1. The data presented in Figure 1 represent the protein concentration in cell homogenates which were not subjected to any extraction procedures.

The proteins in the soluble and polymerized tubulin fractions were separated by electrophoresis and proteins were transferred to PVDF membranes. The membranes were incubated with anti-tubulin antibodies followed by incubation with ^{125}I-labeled second antibody. After washing, the membranes were exposed to X-ray films and the autoradiographs developed. These autoradiographs were then scanned with a laser densitometer.

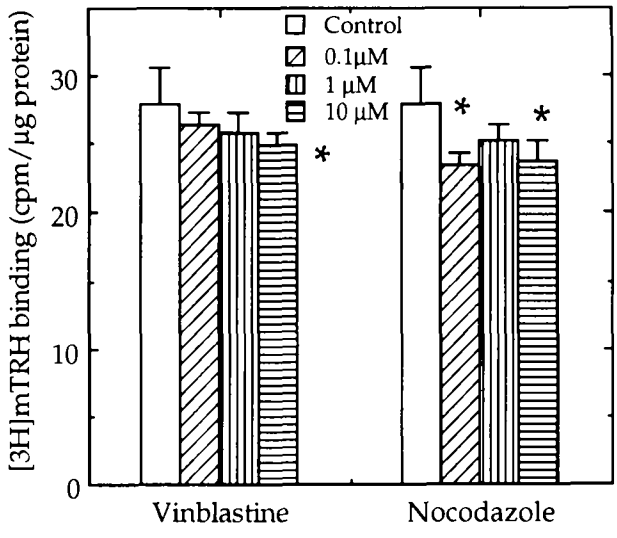

Figure 7 Effect of vinblastine and nocodazole on [^{3}H]TRH binding to GH_3 cell membranes. GH_3 cell membranes (58 μg) were preincubated with various concentrations vinblastine or nocodazole for 2 h on ice and then incubated with 30 000 c.p.m. of [^{3}H]TRH for 4 h on ice. Samples were filtered through glass fiber filters and the radioactivity bound to filters was counted. Each point represents the mean ± SEM of three determinations. Asterisk (*) indicates value significantly different from the control value ($P<0.05$). One of two similar experiments is shown

Because the drug treatment resulted in a decrease in the protein concentration in soluble and polymerized tubulin fractions, the values obtained after scanning the autoradiographs had to be corrected for the changes in the protein pools. Compared to control cells, in vinblastine and nocodazole treated cells, polymerized tubulin levels decreased by 46 and 55%, respectively. Moreover, soluble tubulin levels

increased concomitantly by 85 and 48% in vinblastine and nocodazole treated cells, respectively (Figure 8A and B). In order to determine whether the inhibitory effect of drugs was reversible, cells were incubated for 24 h with the drugs, washed and incubated in drug-free medium and the concentration of the tubulin pools determined at 24 h after drug withdrawal. Drug-treated cells did not recover the ability to form microtubules as indicated by the depressed levels of polymerized tubulin. In vinblastine and nocodazole treated cells, polymerized tubulin levels were 58 and 38% of control, respectively (data not shown).

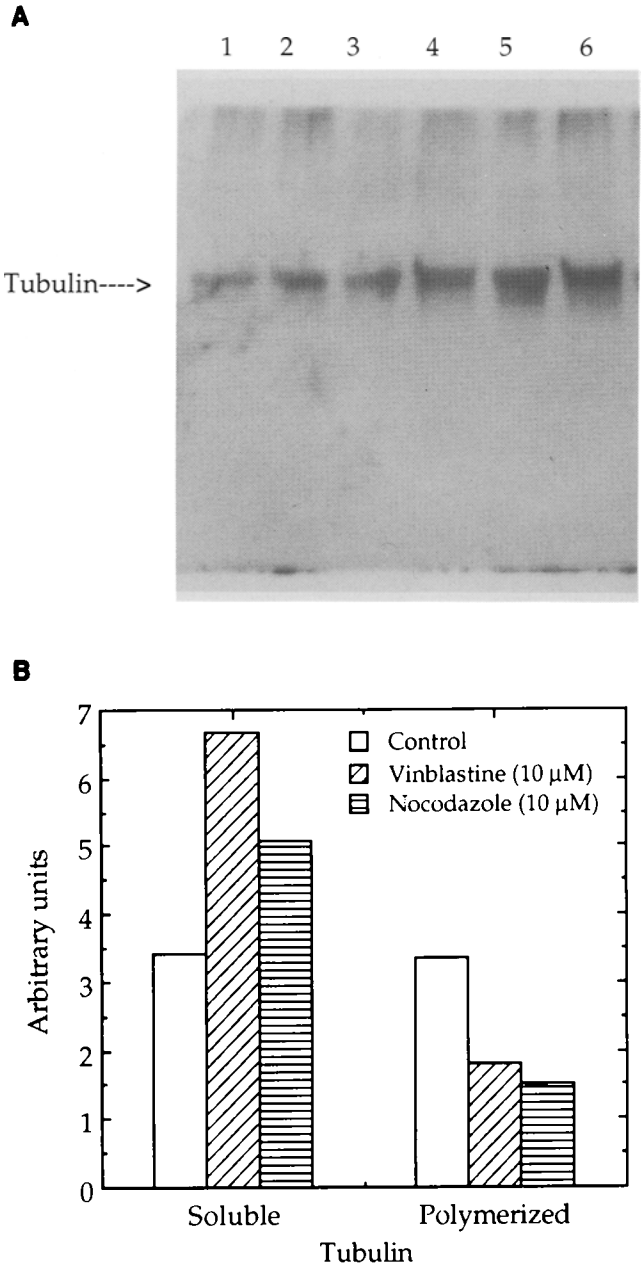

Figure 8 Immunoblot of tubulin in GH_3 cells. GH_3 cells were incubated with 10 μM vinblastine or nocodazole at 37°C for 24 h; after the incubation soluble tubulin (ST) and polymerized tubulin (PT) fractions were prepared. Four μg protein from control or drug-treated samples were separated on SDS-PAGE and the proteins were transferred to PVDF membranes. PVDF membranes were incubated with polyclonal anti-tubulin antibodies followed by incubation with ^{125}I-labeled second antibody. After washing, the membranes were exposed to X-ray films and the autoradiographs developed. One of two similar experiments is shown. (**A**) Lane 1-Control ST; Lane 2-Control PT; Lane 3-Nocodazole ST; Lane 4 Nocodazole PT; Lane 5-Vinblastine ST; Lane 6-Vinblastine PT. (**B**) Tubulin levels determined by laser densitometry. Autoradiographs were analysed by laser densitometry and tubulin levels expressed as arbitrary units. Because the drug treatment resulted in a decrease in the protein concentration in soluble and polymerized tubulin fractions, the values obtained after scanning the autoradiographs were corrected for the changes in the protein pools

Discussion

In the present study, we have observed for the first time that vinblastine inhibits basal and TRH-stimulated PRL secretion by GH_3 cells. Intravenous administration of standard doses of vinblastine (6–18 mg/m^2) results in a peak plasma concentration of 0.4 μM of the drug; doses above 20 mg/m^2 appear to be toxic (reviewed in Rowinsky & Donehower, 1991). In the present study, the suppression of basal and TRH-stimulated PRL secretion by vinblastine occurred at 0.1 μM and 1 μM, respectively. Although it appears that *in vitro* concentrations used in the present study correspond well to the therapeutic doses, the effective concentration in the cell might be much higher compared to that in the medium. In HeLa cells, about 0.1–0.4 μM of vinblastine is concentrated when the drug concentration in the growth medium is between 2 and 10 nM (Wilson & Jordan, 1994).

Our observations are similar to those reported with intact pituitary gland. It has been found that direct injection of vinblastine into the pituitary glands of lactating rats induced the formation of microtubular paracrystals and a concomitant accumulation of PRL secretory granules in the cytoplasm of lactotrophs (Shiino & Rennels, 1975). In addition, incubation of rat anterior pituitary glands with vinblastine resulted in the formation of microtubular paracrystals in somatotrophs and lactotrophs, and was accompanied by an inhibition in PRL secretion (Labrie *et al.*, 1973). Our results with nocodazole are also in agreement with a previous observation, using indirect immunofluorescence, that microtubules in GH_3 cells were completely depolymerized after incubating with nocodazole. In addition, these changes in the microtubules were accompanied by an accumulation of PRL-containing structures, presumably PRL secretory granules, in the cytoplasm of drug-treated cells (Skoufias *et al.*, 1990). However, unlike the present study, PRL secretion was not determined.

In the present study, basal PRL secretion was significantly inhibited by about 50% by 100 nM vinblastine, whereas as high as 10 μM nocodazole was required to achieve a statistically significant inhibition. Furthermore, 1 μM vinblastine significantly suppressed TRH-stimulated PRL secretion, whereas 10 μM nocodazole was required to observe a similar effect. These results suggesting that vinblastine is more potent compared to nocodazole, are consistent with previous observations that a 50% inhibition of the growing rate of microtubules was inhibited by 0.5 μM vinblastine and 3 μM nocodazole. Moreover, microtubule mass in HeLa cells was decreased by 50% by 11 nM vinblastine, whereas 600 nM nocodazole was required to produce an equivalent decrease in microtubule concentration (Wilson & Jordan, 1994).

A decrease in the polymerized tubulin levels in drug-treated cells was accompanied by a concomitant increase in the soluble (i.e. depolymerized) tubulin levels. In addition, exposure of cells to 10 μM vinblastine caused a 25% increase in the total amount of tubulin (soluble + polymerized); total tubulin was 6.76 and 8.48 in control and vinblastine-treated cells, respectively. These observations corroborate the previous findings from Kirschner's laboratory (Cleveland *et al.*, 1981). In view of the fact that higher concentrations of vinblastine cause the formation of microtubular paracrystals, Cleveland *et al.* (1981) suggested that the regulation of tubulin pools occurs in response to changes in the amount of soluble tubulin.

The inhibitory effect of the drugs on hormone secretion and microtubules was not readily reversible. GH_3 cells treated with drugs for 24 h and transferred to drug-free medium were viable for as long as 8 days after drug removal; viability of the cells was assessed by the trypan blue exclusion

method. Moreover, the observation that the drug-treated cells secreted considerable amounts of PRL after washout, albeit to a lesser extent compared to control cells, suggests that the cells are viable after 24 h exposure to the drugs. However, their ability to respond to TRH was not restored even after 48 h after drug removal. The present findings differ from those observed previously with purified tubulin. Nocodazole-induced depolymerization of microtubules was found to be reversible by dialysis of the drug protein complex (Hamel, 1990), and vinblastine binding to tubulin was observed to be reversible when the drug was removed (Wilson & Jordan, 1994). Moreover, the effect of 4 μM nocodazole on GH_3 cells was observed to be reversible. After removal of nocodazole, the normal microtubule network reappeared, and this was accompanied by a reduction in PRL-containing bodies within the cytoplasm (Skoufias *et al.*, 1990). In this study (Skoufias *et al.*, 1990) GH_3 cells were exposed to nocodazole for 4 h, whereas, in the present study the cells were exposed to the drug for 24 h. Exposure of cells to either vinblastine or nocodazole for 4 h did not result in an inhibition of PRL secretion. It appears that the ability of the cells to recover from the drug effect may be dependent upon the concentration of the drug used and the period of time the cells are exposed to vinblastine or nocodazole.

Vinblastine and nocodazole inhibited [^{3}H]TRH binding to GH_3 cell membranes by about 15%. This partial inhibition of TRH binding could not account for the suppression of TRH-stimulated PRL secretion in the presence of these drugs. The results suggest that, in addition to partically blocking TRH binding, these compounds act at a site beyond hormone-receptor interaction to bring about their inhibition of TRH-stimulated PRL secretion. This conclusion is supported by the observation that there are no spare receptors for TRH in GH_3 cells (Cubitt *et al.*, 1990).

Although the inhibition of basal and TRH-stimulated PRL secretion by the drugs was accompanied by a depolymerization of microtubules, the conclusion that these agents acted on PRL secretion only by destroying microtubules or preventing their polymerization is perhaps not entirely accurate. Therefore, in addition to disrupting microtubules, the possibility that vinblastine and nocodazole disrupt any one of the steps in TRH-induced signal transduction pathway (Gershengorn, 1989; Hinkle, 1989; Aragay *et al.*, 1992; Hsieh & Martin, 1992) should be investigated.

Prolactinomas are the most common of the pituitary tumors. Women with these tumors exhibit amenorrhea, oligomenorrhea (or normal periods with infertility), galactorrhea, decreased libido, vaginal dryness and dyspareunia, and delayed menarche. In addition, reduced bone density has been noted in women with prolactinomas. In men decreased libido, impotence, galactorrhea, reduced growth of facial and body hair, small soft testes, apathy, and weight gain have been noted. Currently, patients with prolatinomas are managed by surgery, radiotherapy, or bromocriptine therapy (Levy & Lightman, 1994). However, the recurrence of hyperprolactinemia was noted several years after surgery or after bromocriptine withdrawal, suggesting that new avenues for the management of prolactinomas should be explored. That vinblastine suppresses basal and TRH-stimulated PRL secretion is encouraging and suggests that the effect of this drug should be tested on an *in vivo* model.

Materials and methods

Materials

Thyrotropin releasing hormone (TRH; catalog no. P-2161), methyl TRH (catalog no. P-5173), vinblastine (catalog no. V-1377), nocodazole (catalog no. M-1404) were purchased from Sigma Chemical Co. (St. Louis, MO). Radiolabeled TRH ([^{3}H]methyl TRH; 62.8 Ci/mmol), [^{125}I]goat anti-rabbit IgG F(ab')$_2$ fragment (8.2 μCi/μg) and [^{125}I]PRL (35.2 μCi/μg) were purchased from DuPont-NEN (Wilmington, DE). All other chemicals used in the present study were purchased from Sigma.

Incubation of GH_3 cells with vinblastine and nocodazole

GH_3 cells were maintained at a density of 10^6 ml in T-150 flasks containing Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37°C.

GH_3 cells were transferred to 6-well, flat bottom plates (Falcon, Catalog no. 3046, Beckton Dickinson, Lincoln Park, NJ) where they were grown to a density of 10^6 per well, representing approximately 80% confluency. Just before the addition of vinblastine or nocodazole, the cells were washed twice with serum free medium containing 0.25% BSA, and subsequently incubated with the same medium containing the drugs. After the incubation, medium was collected and kept frozen at −70°C until further analysis. The cells were washed twice with serum free medium containing 0.25% BSA, challenged with 100 nM TRH, and medium collected and kept frozen at −70°C until further analysis.

GH_3 cells were incubated with 10 μM vinblastine or nocodazole for 24 h and the cell viability was tested by trypan blue exclusion. Trypan blue exclusion was 96–100% in control and drug-treated cells.

The concentration of prolactin (PRL) in the medium was determined by a double antibody radioimmunoassay using National Hormone and Pituitary Program reagents and procedures. Determinations were made in duplicate at two dilutions and all samples from an experiment were measured in one assay. The antibody to prolactin was NIDDK-rPRL-1-6 and NIDDK-rPRL-RP-3 served as the reference standard.

Preparation of membranes

Monolayers of GH_3 cells from 15–20 flasks were rinsed twice with homogenizing TED buffer (5 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml leupeptin, and 0.5 mg/ml aprotinin), and scraped into 5 ml of TED buffer. Cells were allowed to swell for 10 min on ice and then were homogenized using a motor-driven Teflon-glass tissue grinder. Cell homogenates were centrifuged at 800 *g* for 10 min. The supernatant was then centrifuged at 100 000 *g* for 60 min. Membrane pellets were resuspended in TED buffer at a concentration of 2–3 mg/ml, and aliquots were kept at −70°C until further use. Protein content was estimated using bovine serum albumin (BSA) as the standard (Bradford, 1976).

[^{3}H]TRH binding to GH_3 cell membranes

The binding of [^{3}H]methyl TRH to GH_3 cell membranes was determined using a rapid filtration procedure. Cell membranes (50–100 μg protein) were incubated in a buffer containing 20 mM Tris-HCl, 2 mM $MgCl_2$ (pH 7.4), and 30 000 c.p.m. of [^{3}H]methyl TRH for 4 h at 4°C. Samples were diluted with ice-cold buffer (20 mM Tris-HCl, 2 mM $MgCl_2$, 100 mM NaCl) and vacuum filtered through glass fiber filters (no. 32, Schleicher and Schuell Inc., Keen, NH). The filter were placed in a scintillation counting vial, mixed with 5 ml of Scintiverse BD (Fisher Scientific, Pittsburgh, PA) and the radioactivity content determined using a Beckman (Palo Alto, CA) liquid scintillation counter. Non-specific binding determined in the presence of 10 μM methyl TRH was 3.4 ± 0.3% (mean ± SD of four experiments, each experiment was conducted in triplicate). Specific binding was 6.5 ± 1% (mean ± SD of four experiments, each experiment was conducted in triplicate).

Preparation of soluble and polymerized tubulin fractions

Cells were homogenized at room temperature in MES buffer (100 mM morpholinoethane sulfonic acid, 2 mM $MgCl_2$,

50 mM KCl and 4 mM EGTA, pH 6.8) with a motor-driven Teflon pestle (Kontes, Vineland, NJ). The homogenate was spun at 100 000 *g* for 60 min at 27°C. The supernatant contained monomeric tubulin and was designated the 'soluble tubulin fraction' and stored at −70°C until further use. The pellet was resuspended in MES buffer (pH 6.8), and kept at 4°C for 60 min. Exposure to cold temperatures results in the depolymerization of microtubules present in the pellet. The suspension was then spun at 100 000 *g* for 60 min at 4°C, and the supernatant was designated the 'polymerized tubulin fraction' and stored at −70°C until further use (Ravindra & Grosvenor, 1988). Protein content was estimated in both the fractions using bovine serum albumin (BSA) as the standard (Bradford, 1976).

Immunoblot analysis of tubulin levels

After electrophoresis (Laemmli, 1970), proteins were transferred to PVDF membranes (BIO-RAD Laboratories, Richmond, CA) using a Trans-blot apparatus (BIO-RAD). The membranes were incubated with polyclonal anti-tubulin antibodies (Chemicon, CA) followed by incubation with ^{125}I-labeled second antibody. After washing, the membranes were exposed by X-ray films and the autoradiographs developed.

Data analysis

Statistical differences among various groups were determined by analysis of variance and Fisher tests using the Statview II program on a Macintosh IIci computer.

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References

Aragay, A.M., Katz, A. & Simon, M.I. (1992). *J. Biol. Chem.*, **267**, 24983–24988.
Bradford, M.M. (1976). Anal. Biochem., **72**, 248–254.
Cleveland, D.W., Lopata, M.A., Sherline, P. & Kirschner, M.W. (1981). *Cell*, **25**, 537–546.
Cubitt, A.B., Geras-Raaka, E. & Gershengorn, M.C. (1990). *Biochem. J.*, **271**, 331–336.
Gershengorn, M.C. (1989). *Ann. New York Acad. Sci.*, **553**, 191 204.
Hamel, E. (1990). *Microtubule Proteins*. Avila, J. (ed). CRC Press: Boca Raton, Florida. pp 89–191.
Hinkle, P.M. (1989). *Ann. New York Acad. Sci.*, **553**, 176 187.
Hsieh, K.P. & Martin, T.F.J. (1992). *Mol. Endocrinol.*, **6**, 1673–1681.
Labrie, F., Gauthier, M., Pelletier, G., Borgeat, P., Lemay, A. & Gouge, J.-J. (1973). *Endocrinology*, **93**, 903–914.
Laemmlie, U.K. (1970). *Nature*, **227**, 680–685.
Levy, A. & Lightman, S.L. (1994). *British Med. J.*, **308**, 1087 1091.
Ravindra, R. & Grosvenor, C.E. (1990). *Mol. Cell. Endocrinol.*, **71**, 165–176.
Ravindra, R. & Grosvenor, C.E. (1988). *Endocrinology*, **122**, 114 119.
Rowinsky, E.K. & Donehower, R.C. (1991). *Pharmac. Ther.*, **52**, 35–84.
Shiino, M. & Rennels, E.G. (1975). *Am. J. Anat.*, **144**, 399–405.
Skoufias, D.A., Burgess, T.L. & Wilson, L. (1990). *J. Cell. Biol.*, **111**, 1929–1937.
Tashjian, A.H. Jr. (1979). *Meth. Enzymol.*, **58**, 527–534.
Wilson, L. & Jordan, M.A. (1994). *Microtubules*. Hyams, J.S. & Lloyd, C.W. (eds). Wiley-Liss: New York, pp 59–83.