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# Effects of the protein tyrosine kinase inhibitor, herbimycin A, on prolactin gene expression in $GH_3$ and 235-1 pituitary tumor cells

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**Abstract**

The high basal level of prolactin (PRL) gene expression in rat pituitary $GH_3$ cells is maintained through the spontaneous activity of voltage-sensitive calcium channels (VSCCs). This can be observed experimentally by addition of 0.5 mM $CaCl_2$ to $GH_3$ cells cultured in a low calcium, serum-free medium. $CaCl_2$ specifically induces PRL gene expression and this induction is inhibited by VSCC blockers. PRL gene expression is also stimulated by several hormones and growth factors. In the present study, we examined the effects of tyrosine kinase inhibitors on the ability of $CaCl_2$, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and thryrotropin-releasing hormone (TRH) to increase PRL mRNA levels. Of several PTK inhibitors used, one PTK inhibitor, herbimycin A, specifically inhibited the $CaCl_2$-induced increase in cytoplasmic and nuclear prolactin (PRL) mRNA without affecting cell viability, cell-cell and cell-matrix adhesion, or the expression of several other genes. The effects of herbimycin A were reversible. In cells pretreated with herbimycin A, PRL mRNA levels were reduced by $69 \pm 12\%$ ($P < 0.001$; $n = 4$).Western blot analysis using anti-phosphotyrosine antibody revealed a decrease of $91 \pm 1\%$ ($P < 0.001$; $n = 4$) in the phosphotyrosine content of proteins in the molecular weight range of 130–160 kDa. After changing the medium back to SFM plus 0.5 mM $CaCl_2$, levels of PRL mRNA increased over a period of several hours, and this increase was accompanied by the tyrosine phosphorylation of two or more proteins in the approximate size range of 130–160 kDa. Herbimycin A also inhibited PRL gene expression in the independently-derived 235-1 lactotrope cell line and lowered the tyrosine specific phosphorylation of protein(s) in a similar size range. Herbimycin A inhibited the ability of bFGF, EGF and TRH to stimulate PRL gene expression in $GH_3$ cells. Again, in cells pretreated with herbimycin A, bFGF induced a reappearance of tyrosine-specific phosphorylation, followed by a reappearance of PRL mRNA. These findings provide evidence for a role for at least one PTK which is necessary for basal and stimulated PRL gene expression. © 1997 Elsevier Science B.V.

*Keywords:* Herbimycin A; Protein tyrosine kinase; Prolactin; Lactotrope; Calcium; EGF; TRH; bFGF

## 1. Introduction

Extracellular $CaCl_2$ can maintain a high basal level of PRL gene expression in primary rat pituitary cultures and pituitary tumor $GH_3$ cells in the absence of serum, growth factors and hormones [1,2]. Culture of $GH_3$ cells in a calcium-free, serum-free medium (SFM) results in a low level of prolactin (PRL) and growth hormone (GH) gene expression. Subsequent addition of 0.5 mM $CaCl_2$ induces a large (5- to 10-fold) and specific increase in PRL mRNA levels

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[1–3]. Pharmacological studies have implicated L-type voltage-sensitive $Ca^{2+}$ channels (VSCCs) and calmodulin in this response [4–7], whereas protein kinase C does not appear to be involved [8,9].

Studies on other cell types have established a relationship between protein tyrosine kinases (PTKs) and the regulation by extracellular $CaCl_2$ of a cellular response. For example, the induction of mouse keratinocyte differentiation by calcium requires activation of the nonreceptor PTK, *fyn*, and at least one other PTK [10]. In neuronal PC12 cells, the VSCC agonist, BAY K8644, activates MAP kinase via a Ras-dependent pathway [11]. The present study was undertaken to investigate whether PTKs were required for the maintenance of PRL gene expression by extracellular $CaCl_2$ in two independently-derived pituitary tumor cell lines. We report here that the PTK inhibitor, herbimycin A, specifically and reversibly represses $CaCl_2$-supported PRL gene expression in GH3 and 235-1 cells. Herbimycin A also specifically blocked the ability of bFGF, EGF and TRH to increase PRL gene expression. These studies provide evidence for at least one PTK which is necessary for basal and stimulated PRL gene expression.

## 2. Materials and methods

The cloned probes for PRL, GH, and glucose-regulated protein 78 (GRP78) were described previously [2,3]. The glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA clone [12] was provided by Dr. David Rowe (University of Connecticut Health Center, Farmington, CT). $GH_3$ cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in suspension culture as described [13]. The 235-1 cell line was generously donated by Dr. H.H. Samuels (NYU Medical Center, New York, NY) and was maintained in monolayer as described [14]. At the time of an experiment, cells were centrifuged out of growth medium and resuspended in serum-free medium (SFM). Although SFM has no added $CaCl_2$, it contains about 50 $\mu$M of contaminating $CaCl_2$. Culturing cells in SFM does not drastically lower intracellular $Ca^{2+}$, but does allow one to observe the effects of the VSCC on PRL gene expression by adding 0.5 mM $CaCl_2$. $GH_3$ cells cultured in SFM show greater than 90% viability for several days, and respond to stimulators of either PRL or GH gene expression [2,13]. The exact treatment for each experiment is described in the legend to the corresponding figure. Herbimycin A, lavendustin A, and methyl-2,5 dihyroxycinnamate were purchased from Life Technologies (Gaithersburg, MD), and were diluted in PBS from a DMSO stock solution. Genistein, daidzein, and RG-13022 were obtained from BIOMOL (Plymouth Meeting, PA). Basic FGF, EGF and TRH were obtained from Sigma (St. Louis, MO).

### *2.1. Analysis of mRNA levels by Northern blot hybridization*

Preparation of cytoplasmic mRNA for analysis by either Northern blot or RNA dot hybridization was performed as described [15] except that the spin step with vanadyl-ribonucleoside complexes was omitted. For the preparation of nuclear RNA, GH3 cells were collected, pooled, washed once in PBS, and then resuspended in 0.9 ml of PBS. Cells were lysed by addition of 0.5% NP40, incubated on ice for 3 min, then centrifuged at $1000 \times g$ for 5 min at 4°C. The nuclear RNA was isolated by guanidine extraction and centrifugation through CsCl [14]. The relative levels of specific mRNAs were measured by Northern blot hybridization using 1% agarose/formaldehyde gels as described [3]. Gels were stained prior to transfer with ethidium bromide in order to measure relative mobilities of 28 s and 18 s ribosomal RNAs, and to ascertain even loading. The DNA probes were labeled with $^{32}$P-dCTP using the Nick-Translation Kit N5000 (Amersham, Arlingtin Heights, IL) or by the RTS RadPrime DNA labeling system (Life Technologies, Gaithersburg, MD), followed by purification using MicroSpin S-200 HR columns (Pharmacia, Piscataway, NJ).

### *2.2. Western blot analysis of phosphotyrosyl-containing proteins*

For phosphotyrosine immunoblotting, samples were electrophoresed on either 8% or 10% SDS-polyacrylamide gels as indicated. For analysis of whole cells, the medium was removed and the cells (plated at equal densities for each treatment) were dissolved by scraping with a Teflon policeman in 250 ml of

boiling Laemmli sample buffer [16]. The samples were then boiled for 5 min and the DNA was sheared by passing 5 times through a 26 gauge needle. Insoluble material was removed by centrifuging the cell lysates for 5 min in a microcentrifuge at $10\,000 \times g$. Prior to electrophoresis, cell extracts were diluted and the protein concentration of each sample was determined [17]. Typically, 100 $\mu$g of protein was electrophoresed. Nitrocellulose sheets were blocked with 10% bovine serum albumin (BSA, Boehringer Mannheim, Indianapolis, IN) in Tris-buffered saline (100 mM NaCl, 50 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 overnight with agitation at room temperature. After removal of blocking solution, antiphosphotyrosine antibody (Upstate Biotechcnology, Lake Placid, NY) at a dilution of 1:1000 in TBS containing 3% BSA was added and the blots were incubated at room temperature with agitation for 1 to 2 h. Horseradish peroxidase conjugated sheep anti-mouse antibody (Kirkegaard and Perry, Inc., Gaithersburg, MD) was used at a dilution of 1:25 000 as a secondary antibody and reactivity was determined by chemiluminescence (Kirkegaard and Perry) according to manufacturers instructions.

### *2.3. Quantification of autoradiograms and statistical analysis*

Autoradiograms were scanned with an Apple One Scanner using Ofoto scanning software (Apple Computers, Cupertino, CA) and quantified using IP Lab Gel software (Signal Analytics Corp., Vienna, VA). Alternatively, blots were analyzed by a Betascope blot anaylzer (Betagen) or by phosphoimaging utilizing a Phosphoimager SI (Molecular Dynamics, Sunnyvale, CA) and IP Lab Gel software. The effects of herbimycin A on RNA levels or phosphotyrosine content were analyzed by Student's *t* test.

## 3. Results

### *3.1. Herbimycin A specifically blocked the ability of extracellular $CaCl_2$ to maintain high PRL mRNA levels in somatolactotrope $GH_3$ cells*

We first screened the effects of several PTK inhibitors on the ability of 0.5 mM $CaCl_2$ to return PRL gene expression to a high basal level in $GH_3$ cells cultured in SFM. The following inhibitors were examined: genistein, lavendustin A, erbstatin analogue, the tyrophostin RG-13022 and herbimycin A. Genistein and herbimycin A reduced PRL mRNA levels by approximately 80% (Fig. 1). Lavendustin A and erbstatin analogue also decreased PRL mRNA to a lesser extent than genistein and herbimycin A. RG-13022 had no effect. The inhibitory effects on PRL mRNA levels of lavendustin A and erbsatin analogue, and to a lesser extent genistein, were accompanied by a noticable decrease in cell viability, as well as cell-cell and cell-matrix adhesion (data not shown). In contrast, herbimycin A strongly and reproducibly inhibited PRL gene expression, but had little or no effect on the expression of several other genes, including growth hormone. Also, in at least 10

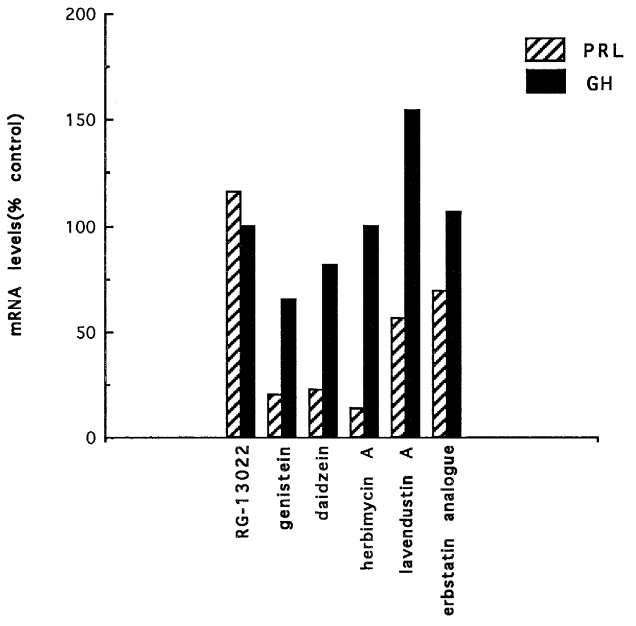


Fig. 1. Effects of various PTK inhibitors on the ability of $CaCl_2$ to increase PRL mRNA levels in $GH_3$ cells in 24 h. The concentration of each inhibitor utilized was as follows: RG-13022 (40 $\mu$M), genistein (27.6 $\mu$M), daidzein (27.6 $\mu$M), herbimycin A (875 nM), lavendustin A (25 $\mu$M), and erbstatin analogue (12.5 $\mu$M). Note that erbstatin analogue refers to methyl 2,5-dihydroxycinnamate. PRL and GH mRNA levels were measured by Northern blot or RNA dot hybridization. The percentage values presented for each inhibitor tested were calculated from the mean of duplicate samples relative to $CaCl_2$-treated controls (set at 100%) from one representative experiment. Variation between duplicates was less than 5%, except for RG-13022 values, which varied by 20%.

experiments, herbimycin A had no visible effect on cell viability or general appearance, calcium-induced cell-cell adhesion or adhesion of cells to the tissue culture dish (data not shown). Therefore, herbimycin A was used exclusively for completion of this study.

In a representative experiment shown in Fig. 2A, treatment of $GH_3$ cells with $CaCl_2$ increased PRL by

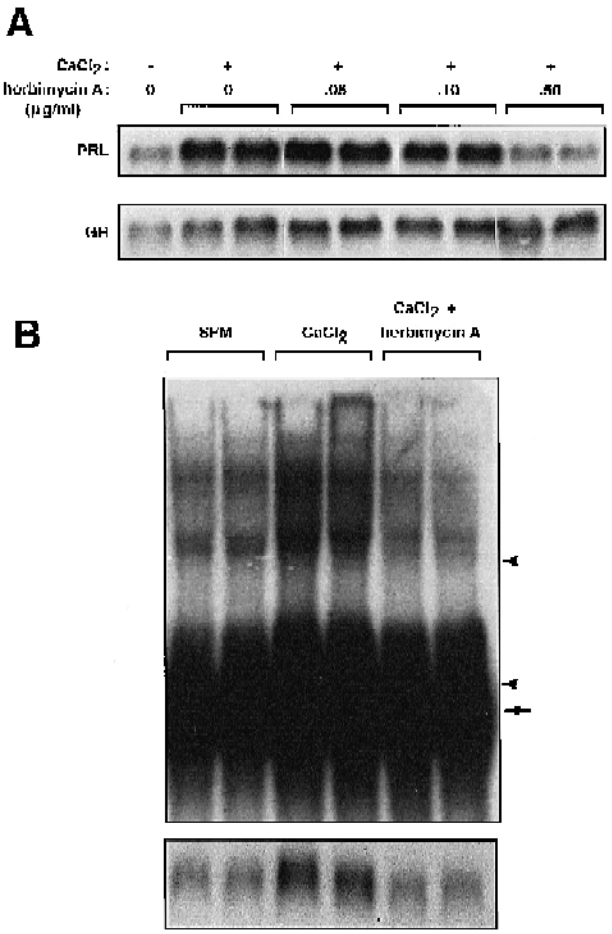


Fig. 2. Herbimycin A inhibits the $CaCl_2$-dependent stimulation of PRL mRNA levels. (A) Cytoplasmic PRL and GH mRNA levels from $GH_3$ cells that were cultured in SFM in the absence and presence of increasing doses of herbimycin A overnight followed by the addition of 0.5 mM $CaCl_2$ for 24 h. (B) Nuclear PRL RNA. $GH_3$ cells were cultured in duplicate in SFM in the absence and presence of 0.25 $\mu$g/ml of herbimycin A overnight followed by the addition of 0.5 mM $CaCl_2$ for 24 h. The upper panel of (B) represents a 1 wk exposure by autoradiography and the lower panel of (B) represents a 1-h exposure on a Betascope blot analyzer of the area of the blot shown in (A) depicted by the arrow. *Arrowheads*, 28S and 18S rRNA bands. *Arrow*, fully-spliced 1 kb-sized PRL mRNA.

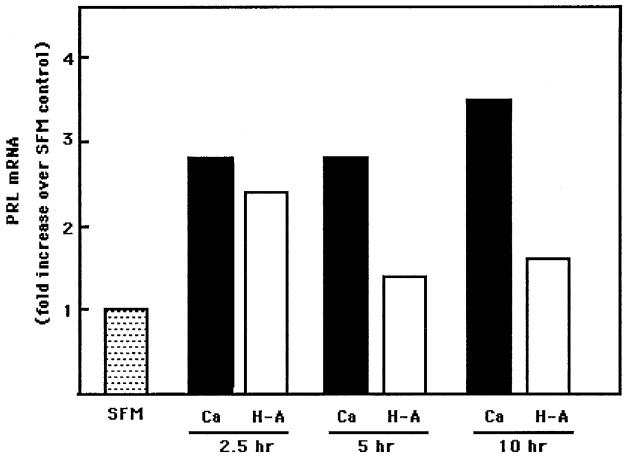


Fig. 3. Effect of herbimycin A on PRL mRNA levels in cells preinduced with $CaCl_2$. $GH_3$ cells were cultured for 24 h in SFM in the absence (SFM control) or presence of 0.5 mM $CaCl_2$. Herbimycin A (0.5 $\mu$g/ml) was then added for 2.5, 5 and 10 h. PRL mRNA was assayed in duplicate samples by Northern blot hybridization and quantified as described in Section 2. Data are presented as the means of values from duplicate cultures. There was less than 10% variation between duplicates.

about 5-fold over SFM controls. Herbimycin A at a concentration of 0.5 $\mu$g/ml completely blocked the effect of $CaCl_2$, reducing PRL mRNA levels to 14% of the $CaCl_2$-induced levels. $CaCl_2$ also induced a small (i.e., 1.5- to 2-fold) increase in GH mRNA. Herbimycin A had no effect on these slightly elevated GH mRNA levels (Fig. 1A). In four separate experiments (each performed with duplicate cultures), herbimycin A significantly ($P < 0.001$) reduced PRL mRNA levels by $69 \pm 12\%$, but did not affect levels of GAPDH mRNA or GRP78 mRNA (data not shown). Herbimycin A reduced both cytoplasmic PRL mRNA (Fig. 2A) and nuclear PRL gene transcripts (Fig. 2B).

Previous studies with herbimycin A indicate that the drug acts by promoting the degradation of specific PTKs [18]. In the experiment shown in Fig. 3, we examined the relative rapidity of the effect of herbimycin A in $GH_3$ cells that had been pretreated overnight in SFM plus 0.5 mM $CaCl_2$. $CaCl_2$ increased PRL mRNA about 3-fold over SFM control. Consistent with previous studies which indicate a relatively slow effect of the drug (18), herbimycin A had essentially no effect after 2.5 h, but depressed PRL mRNA levels by about 50% at 5 h and 10 h.

### 3.2. Effects of herbimycin A on the tyrosine-specific phosphorylation of $GH_3$ cell proteins and PRL gene expression are reversible

The effects of herbimycin A on protein tyrosine phosphorylation were examined by Western blot analysis using an anti-phosphotyrosine antibody. In cells treated overnight with herbimycin A, PRL mRNA levels were undetectable and a low level of apparent phosphotyrosine staining was observed on Western blots (Fig. 4A). This staining was nonspecific, as it was detected in the absence of primary antibody (data not shown). Subsequent to changing the medium back to SFM containing 0.5 mM $CaCl_2$, PRL mRNA levels slowly increased over a 24 h period (Fig. 4A, upper panel). Concomitantly, an increased phosphotyrosine staining of two or more proteins in the range of approximately 130–160 kDa was detected (Fig. 4A, lower panel). The phosphotyrosine staining of these proteins was not observed in the absence of primary antibody (data not shown), nor was it detected in cells that were continually cultured in herbimycin A (Fig. 4B) or in herbimycin A-pretreated cells switched to SFM without CaCl2 for 24 h (lane 1, Fig. 4A).

### 3.3. Effects of herbimycin A on PRL expression and tyrosine phosphorylation in 235-1 lactotropes

The 235-1 cell line was independently derived from $GH_3$ cells, and only produces PRL [19]. PRL expression is also inducible by $CaCl_2$ in these cells [14]. Thus, we examined both the effects of herbimycin A on the $CaCl_2$ induction of PRL and on tyrosine-specific phosphorylation in this lactotrope cell line. The profile of tyrosine-specific protein

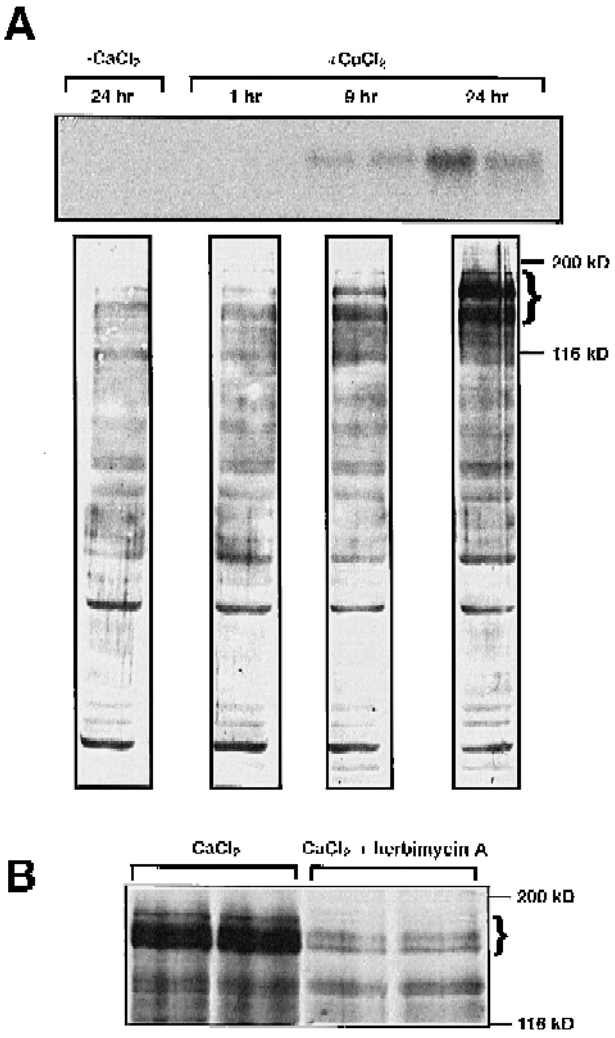


Fig. 4. A. Effects of Herbimycin A on PRL mRNA levels and protein tyrosine phosphorylation are reversible by $CaCl_2$.. $GH_3$ cells cultured in SFM were treated with 0.5 $\mu$g/ml of herbimycin A for 16 h. After medium change, the cells were allowed to recover in fresh SFM for 5 h, and then incubated for 24 h in SFM only (-$CaCl_2$), or in SFM plus 0.5 mM $CaCl_2$ (+$CaCl_2$) for 1 h, 9 h, or 24 h. *Upper panel*, duplicate RNA samples were probed for PRL mRNA by Northern blot hybridization. RNA values increased by at least 10-fold between the 1 and 9 h time points, although exact quantification of this increase is difficult due to the barely detectable signal in the 1 h sample. *Lower panel*, antiphosphotyrosine immunoblot (8% polyacrylamide gel). MW standards are indicated and bracket represents 130-160 kDa complex. Essentially all of the lighter bands represent nonspecific staining that was observed in the absence of primary antibody. Quantification of the lower band within the bracket revealed an 8-fold increase between 24 h samples in the presence and absence of $CaCl_2$. This experiment was repeated twice with similar results. B. Effect of continuous treatment of herbimycin A on tyrosine phosphorylation of $GH_3$ cell proteins in the 130–160 kDal size range. Cells were cultured for 16 h in SFM in the absence or presence of 0.5 $\mu$g/ml of herbimycin A. $CaCl_2$ was then added to all dishes and cells incubated for an additional 24 h. Tyrosine-specific phosphorylation was examined by Western blot using a 10% polyacrylamide-SDS gel. Data from four independent experiments indicated that the phosphotyrosine content of the proteins within the size range indicated by the bracket decreased by $91 \pm 1\%$ ($P < 0.001$).

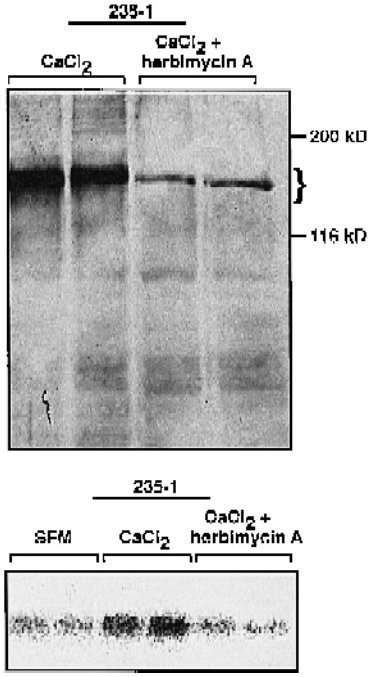


Fig. 5. Effects of herbimycin A on tyrosine-specific phosphorylation (upper panel) and PRL mRNA levels (lower panel, as detected by Betagen Betascope) in 235-1 lactotropes. MW standards are indicated and brackets in (A) and upper panel of (B) denote 150-180 kDa complex. Phosphotyrosine content was reduced by about 90%, whereas PRL mRNA was reduced by about 65%. Duplicate values differed by less than 10%.

phosphorylation was very similar between $GH_3$ and 235-1 cells (compare Fig. 4 to Fig. 5), in that one or more proteins in the molecular weight range of 130–160 kDa were strongly stained with anti-phosphotyrosine antibody. Both tyrosine phosphorylation and the level of PRL mRNA in 235-1 cells were decreased by herbimycin A treatment (Fig. 5).

### *3.4. Effects of herbimycin A on bFGF, EGF and TRH stimulation of PRL gene expression*

The studies described above show that herbimycin A effectively inhibits the high basal level of PRL gene expression, as maintained by extracellular $CaCl_2$, in the absence of serum. PRL gene expression is also regulated by numerous growth factors and hormones. We examined whether herbimycin A blocked three stimulators of PRL gene expression: bFGF, EGF and TRH. As shown in Fig. 6, herbimycin A essentially completely blocked the effects of TRH and EGF, and inhibited the effects of FGF by about 60%.

We next examined whether the effects of herbimycin A could be reversed by bFGF after removal of the drug, similar to the experiment shown in Fig. 4. Again, pretreatment with herbimycin A resulted in loss of detection of both PRL mRNA (Fig. 7, upper panel) and tyrosine-specific phosphorylation (Fig. 7, lower panel). Removal of the drug alone did not reverse these observations (Fig. 7; lane 1). However, addition of bFGF after removal of herbimycin A induced tyrosine phosphorylation by 6.5 h, and increased PRL mRNA by 12 h. The finding that both $CaCl_2$ and bFGF required several hours to increase tyrosine phosphorylation is consistent with previous reports that herbimycin A acts by inducing degrada-

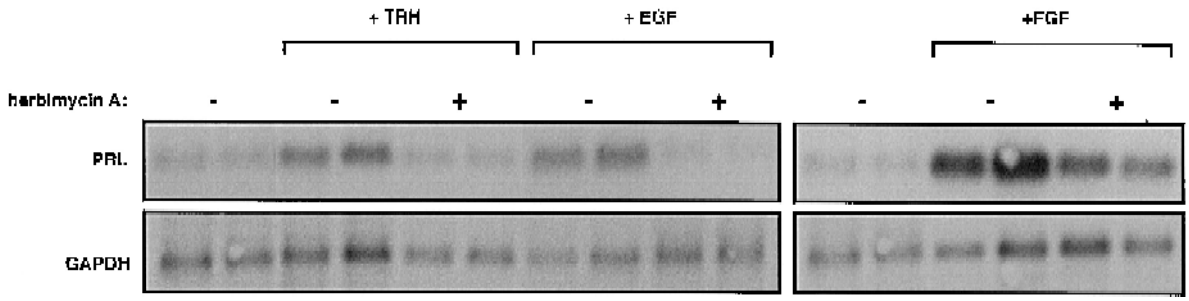


Fig. 6. Herbimycin A inhibits the ability of TRH, EGF and FGF to increase PRL mRNA levels. Cells were treated essentially the same as described in Fig. 2, except that cells were induced with either TRH (100 nM), EGF (40 ng/ml) or bFGF (FGF; 40 ng/ml) plus or minus 0.5 $\mu$g/ml of herbimycin A. Note that FGF was examined in a separate experiment, and is shown with its own SFM control. This was repeated twice with the same results.

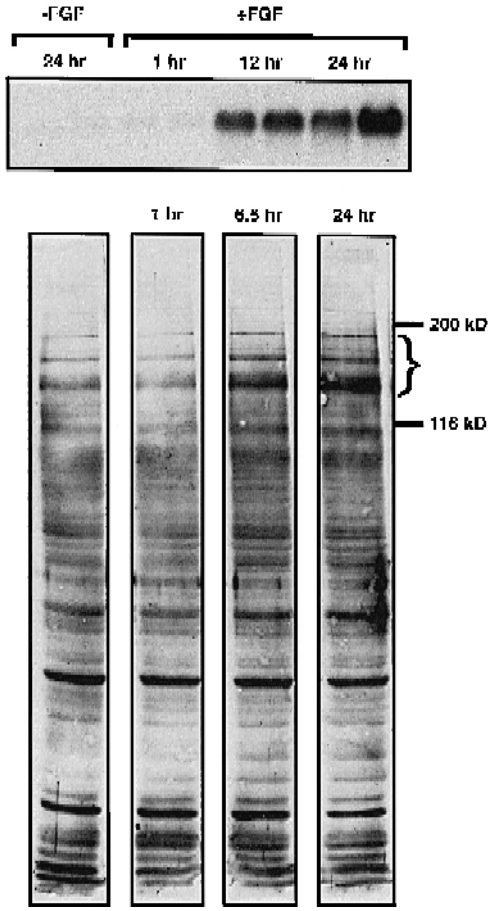


Fig. 7. The effects of herbimycin A are reversible by bFGF. This experiment was performed essentially as described in Fig. 4, except that cells were treated after herbimycin A with 40 ng/ml of bFGF (FGF). Note that, as indicated, shorter time points were used for Western blot analysis than those used for Northern blot analysis.

tion of PTKs [18]. We note that in $GH_3$ cells not pretreated with herbimycin A, $CaCl_2$ and bFGF induce changes in tyrosine phosphorylation patterns within minutes ([20], and data not shown).

## 4. Discussion

Herbimycin A is a benzoquinonoid ansamycin antibiotic that reverses the morphology of various cells transformed by oncogenic PTKs (e.g., Src, Yes, Fps, Ros, Abl, Ret, and Erb B) but not by oncogenes which do not posssess PTK activity (e.g., Myc, Raf and Ras; [21,22]). Herbimycin A specifically reduces the PTK activity of these oncogene products and thereby lowers the extent of tyrosine phosphorylation of cellular proteins [22]. Herbimycin A also alters the differentiated phenotype of various cells [22]. In the human chronic myelogenous leukemia K562 cell line expressing a mutated c-Abl with enhanced PTK activity, herbimycin A induced erythroid differentiation concomitant with a reduced tyrosine phosphorylation of the c-Abl kinase [23]. Herbimycin A is also capable of triggering endoderm differentiation in embryonal carcinoma F9 cells, and terminal erythroid differentiation of erythroleukemic MEL cells [24]. Given the above findings, it is not surprising that herbimycin A modulates specific gene expression. The drug increased fibronectin mRNA levels in RSV-infected rat kidney cells [25], and inhibited c-myc expression in mouse lymphoblastoma L5178Y cells [26].

In this study, we examined whether several PTK inhibitors could block the ability of $CaCl_2$ to induce PRL gene expression. Most of these inhibitors reduced PRL mRNA nonspecifically and/or severly decreased cell viability. Thus, it is likely that these drugs inhibited one or more PTKs which are essential for cell viability. On the other hand, herbimycin A effectively inhibited the $CaCl_2$-induced increase in the levels of cytoplasmic PRL mRNA and nuclear PRL pre-mRNAs. Importantly, this effect of herbimycin A was highly specific for PRL, and was observed in two independently-derived pituitary tumor cell lines. Herbimycin A had no effect on cell viability or adhesion, and was completely reversible.

Although herbimycin A has been shown to inhibit several PTKs, the specificity of its effects in $GH_3$ cells argues that, in this cell type, herbimycin A inhibits one or a few PTK(s) that are specifically linked to PRL gene expression. We detected the presence of one or more proteins in the range of 130–160 kDa whose tyrosine-specific phosphorylation is inhibited by herbimycin A. This effect of herbimycin A can be reversed by both $CaCl_2$ and bFGF in a manner that is closely correlated with the reprise of PRL gene expression. These data support the assumption that herbimycin A elicits its effects on PRL gene expression in $GH_3$ cells through inhibition of one or more PTK(s). However, we have neither determined the identity of the phosphorylated pro-

teins, nor any role of these proteins in the regulation of PRL gene expresson.

The finding that the reversal of herbimycin A-induced effects took several hours is consistent with the drugs ability to induce selective degradation of some of its targets (e.g., lck [18]). Also, the finding that either $CaCl_2$ or bFGF was required for this reversal indicates that these stimulators of PRL gene expression may also promote the synthesis of a specific PTK, possibly as part of their ability to induce or maintain a lactotropic phenotype. In this light, it is interesting to note that the expression of other PTK genes is stimulated over a period of 24–48 h upon hormonal or growth factor induction of differentiation in other cell types (e.g. [27]). Also, it is worth noting that factors which acutely stimulate PRL gene expression (e.g., FGF, EGF, NGF) have also been shown to induce other features of the lactotrope phenotype over a peroid of several days ([28–30]; our unpublished observations). Thus, one explanation for the ability of herbimycin A to block several stimulatory factors is that $GH_3$ cells may express a PTK which plays a role in the maintenance of PRL gene expression as part of the differentiated function of these cells. Such a PTK could be required for PRL gene expression, but not necessarily be a component of the signal transduction pathways of $CaCl_2$, FGF, EGF and TRH.

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