Changes in numbers of prolactin receptors during the cell cycle of Nb2 cells

I G Camarillo and J A Rillema

Department of Physiology, Wayne State University School of Medicine, 540 East Canfield, Detroit, Michigan 48201, USA

(Requests for offprints should be addressed to J A Rillema)

Abstract

Lactogenic hormones including prolactin (PRL) have mitogenic effects on Nb2 cells, a pre-T lymphoma cell line. Previous studies have characterized the PRL stimulation of cellular processes such as RNA/DNA synthesis, signaling molecule activation, and the expression of specific genes. The data presented here explores the fluctuations in plasma membrane PRL receptor (PRLR) number that occur in the Nb2 cells during the course of a 24 h cell cycle. PRLR abundance was determined by measuring specific binding of [125 I] oPRL to G₁ arrested-intact Nb2 cells in which the cell cycle was initiated by addition of nonradioactive oPRL. Preliminary

studies revealed that 1 ng/ml oPRL was the minimum PRL concentration that causes a maximal stimulation of mitogenesis, without interfering with [125 I] oPRL binding measurements. Subsequent experiments revealed that upon cell cycle initiation of G₁ arrested Nb2 cells with 1 ng/ml oPRL, PRLR number remained constant for the initial 6 h. After 8 h PRLR numbers decreased and at 12 h, the PRLR number was less than 25% of the initial value. After 12 h, PRLR numbers increased and reach initial values by 18 hr. These studies show that the expression of cell surface PRL receptors is modulated in a sequential fashion during the cell cycle of Nb2 cells.

Introduction

It is well known that progression of Nb2 node lymphoma cells through the cell cycle line is stimulated by lactogenic hormones (Gout *et al.* 1980, Gout *et al.* 1985, Noble *et al.* 1980). However, the molecular signaling pathways by which PRL elicits its mitogenic effect have only been partially characterized. An intermediate form of the PRLR, a member of the cytokine receptor family (Ali *et al.* 1991, Kelly *et al.* 1988), is expressed in the Nb2 cells. It is assumed that the PRL responses in the Nb2 cells are elicited via signaling through this receptor. The Nb2 cell line is exquisitely responsive to prolactin, and is therefore a useful model for determining signaling processes involved in growth factor stimulated cell division. Early studies with the Nb2 cells characterized the onset of PRL induced increases in protein, DNA, and RNA synthesis (Richards *et al.* 1982, Rillema *et al.* 1989) as well as increases in ornithine decarboxylase activity (Buckley *et al.* 1986, Richards *et al.* 1982). More recently, studies with Nb2 cells have focused on PRL induced signal transduction pathways. PRL has been shown to activate a host of kinases, linking proteins, and various other signaling molecules within minutes after addition to Nb2 cells (Campbell *et al.* 1994, Clevenger *et al.* 1995, Clevenger *et al.* 1994, Erwin *et al.* 1995, Rao *et al.* 1995).

In the present studies, the main goal was to characterize the changes in PRLR numbers which occur during the 24 h cell cycle of Nb2 cells. This was accomplished by measuring specific binding of [125 I] oPRL to intact Nb2 cells at regular time intervals throughout a 24 h mitogenic cycle. In initial

experiments Nb2 cells were treated for 24 h with increasing PRL concentrations to determine the optimum PRL levels which would result in a maximum rate of cell proliferation but minimum interference with specific [125 I] oPRL binding measurements.

Materials and Methods

Nb2 Cell Culture

The PRL-dependent Nb2 cell line was provided by Dr CT Beer of the Cancer Control Agency of British Columbia (Vancouver, British Columbia, Canada). Cells were grown in Fischer's medium supplemented with 10% fetal calf serum and 10% horse serum (Sigma Chemical Co., St. Louis, MO, USA), 1.0×10^{-4} M 2-mercaptoethanol, 100 I.U./l penicillin, and 100 mg/l streptomycin, (Eli Lilly Co., Indianapolis, IN, USA) as previously described (Gout *et al.* 1985). Incubation for 24 h in serum free media ('stationary media') effectively arrests growth of Nb2 cells at the G₀/G₁ phase of the cell cycle (Rillema *et al.* 1989). After serum depletion, cells are harvested and the cell cycle is initiated by treatment with oPRL (NIH-P-S-17, a gift from the NIAMDD).

Specific [125 I] oPRL Binding

The [125 I] oPRL (160 μ Ci/ μ g) was prepared by the lactoperoxidase method by Dr DM Lawson (Rillema & Lawson 1994). In initial experiments, the optimum concentration of nonradioactive oPRL employed to commence the cell cycle was determined. Groups of synchronized Nb2

cells were harvested by centrifugation at 200 *g* for 10 min, and resuspended in 'stationary medium' at $1\text{--}1.5 \times 10^5$ cells/ml. Next, 900 μl aliquots of cells were dispensed into 24 well plates and increasing concentrations (0–100 ng/ml) of oPRL in 100 μl aliquots were added. Each treatment group contained multiple samples for determining both [^{125}I] oPRL binding and cell number. Cell numbers were determined for all treatment combinations by adding 1 ml cells to 9 ml Hematall isotonic fluid after which the cells were quantitated on a Coulter model ZM counter. Cells were then incubated for 24 h at 37°C in the presence of a 95% air/5% CO_2 gas mixture. After this, cells were washed with 5 ml ice cold phosphate buffered saline (PBS) and collected by centrifugation at 200 *g*. After resuspension in PBS containing either 0.01 $\mu\text{Ci/ml}$ [^{125}I] oPRL, (to measure total binding) or 0.01 $\mu\text{Ci/ml}$ [^{125}I] oPRL plus 10 $\mu\text{g/ml}$ nonradioactive oPRL (to measure nonspecific binding), incubations at 37°C were carried out for 1 h. Next, 1 ml samples were washed through 5 ml ice-cold PBS by centrifugation at 400 *g* for 10 min and the supernatant was decanted. Radioactivity in the pelleted cells was then determined and 'specific binding' was calculated by subtracting 'nonspecific binding' from 'total binding'. After determining the optimum oPRL concentration that results in maximal cell division but minimal [^{125}I] oPRL binding measurement interference, PRLR numbers were determined at 2 h intervals during the 24 h Nb2 cell cycle. Binding measurements were performed employing the procedures described above.

Each of the experiments was repeated three times with 4–6 replicates (as specified in each figure) for each experimental combination. Data were analyzed by an analysis of variance followed by Dunnet's test. Means were considered significantly different with $P < 0.05$.

Results

In order to investigate PRLR content in the Nb2 lymphoma cell line, optimum conditions were first established to effectively measure specific [^{125}I] oPRL binding. The first task was to identify an oPRL concentration which would optimally induce the cell cycle, but would not down-regulate receptor numbers or interfere with radioactive binding measurements. In initial experiments synchronized cells were treated with increasing concentrations of oPRL for 24 h. Subsequently, both specific [^{125}I] oPRL binding and cell numbers were determined. Figure 1 clearly shows that cells treated with oPRL levels exceeding 0.1 ng/ml expressed a maximum rate of mitogenesis. However, when [^{125}I] oPRL binding was determined and expressed as cpm/ 1×10^5 cells, a marked decrease in measurable PRLR numbers is apparent as the nonradioactive oPRL concentration is increased above 1 ng/ml. It is evident from the data in Fig. 2 that when Nb2 cells are exposed to 1.0 ng/ml oPRL for 24 h the greatest amount of specific radioactive binding occurs. In contrast, when

Figure 1 Effect of PRL concentration on cell population. Nb2 cells (1×10^5 /ml) synchronized at the G₀/G₁ interphase were resuspended in stationary media. Increasing concentrations of oPRL were added after which cells were incubated for 24 h and cell population determined. Values represent the means $\pm$ S.E. of four observations.

Figure 2 Effect of PRL concentration on [^{125}I] PRL binding. Synchronized Nb2 cells (1×10^5 /ml) were treated with increasing concentrations of oPRL. After incubation for 24 h specific PRL binding was measured by exposing cells to 0.01 mCi/ml [^{125}I] oPRL for 1 h. Values represent the means $\pm$ S.E. of six observations.

nonradioactive oPRL concentrations exceed 1.0 ng/ml there is virtually no detectable specific [125 I] oPRL binding. Thus, even though maximum mitogenesis occurs with oPRL concentrations above 1 ng/ml, there are no PRLR's present on the cell membrane to interact with [125 I] oPRL. This is likely due to a down-regulation of cell surface receptors on cells exposed to the high oPRL concentrations. In any case, 1 ng/ml oPRL is the optimum oPRL concentration which induces a maximum stimulation of mitogenesis, but causes no receptor down-regulation or interference with the [125 I] oPRL binding assay. In all subsequent experiments, 1.0 ng/ml oPRL was therefore used to initiate the cell cycle in the Nb2 cells.

To investigate possible PRLR level changes during the 24 h Nb2 cell cycle, growth arrested cells were treated with 1.0 ng/ml oPRL. Both cell counts and specific [125 I] oPRL binding were then measured at regular time intervals during the cell cycle. Figure 3 shows that cell numbers begin increasing 18 h after addition of oPRL. PRLR numbers during the cell cycle are presented in Fig. 4. The numbers of receptors decrease precipitously between hours 6 and 14 of the cell cycle; at 12 h the receptor number is less than 25% of that present at the beginning of the cell cycle. The time of the cell cycle when receptor numbers decrease and reach a minimum correlates with the S-phase of the cell cycle (Murphy *et al.* 1988, Richards *et al.* 1982). After completion of the cell cycle after 24 h, PRLR numbers are the same as in the cells that were growth arrested at the G₀/G₁ phase of the cell cycle.

Figure 3 Time course of PRL stimulated Nb2 mitogenesis. Nb2 cells (1.5×10^5 /ml) arrested at G₁ were treated with 1.0 ng/ml oPRL. Cell numbers were counted at regular time intervals through 24 h. Values represent the means $\pm$ S.E. of four observations.

Figure 4 Time course of [125 I] oPRL binding throughout the cell cycle. Nb2 cells (1.5×10^5 /ml) arrested at G₁ were stimulated with 1.0 ng/ml oPRL. Cell samples were taken at regular time intervals throughout the course of a 24 h cell cycle. Cells were exposed to 0.01 mCi/ml [125 I] oPRL for 1 h to determine specific binding. Values represent the means $\pm$ S.E. of six observations.

Discussion

The central focus of these studies was to observe PRLR level changes during the course of the cell cycle of Nb2 cells. For this purpose, 1.0 ng/ml PRL was employed to stimulate the Nb2 cells to enter the cell cycle after they were synchronized at the G₀/G₁ interface. PRL at 1.0 ng/ml was chosen because this concentration of PRL stimulates a maximum rate of mitogenesis but does not interfere with [125 I] oPRL binding to its receptors. Exposure to higher concentrations of oPRL caused a down-regulation of [125 I] binding to its receptors. The significance of this observation is not immediately apparent since PRL at all concentrations above 1.0 ng/ml has been reported to have an equivalent effect on mitogenesis in the Nb2 cells (Gout *et al.* 1980).

The cell cycle data presented here indicate that PRL receptor numbers are maintained during the G₁ phase (8 h) of the cell cycle and subsequently experience a precipitous decrease during the S-phase of the cell cycle, similar to results obtained by Shiu *et al.* (1983). However, unlike Shiu and colleagues, we observed that following the S-phase PRL receptor numbers return to G₁ phase levels by the completion of the cell cycle. The maintenance of receptor numbers during the G₁ phase may be of importance since the continuous presence of PRL for at least the first four h during this phase is required for PRL to subsequently stimulate mitogenesis (Murphy *et al.* 1988). This continuous exposure to PRL in the G₁ phase, however, is not essential for PRL to stimulate entry

into the S-phase of the cell cycle. Although we do not know the significance of the sequential changes in PRL receptor number during the cell cycle, the required continuous presence of PRL during the G₁ phase necessary for subsequent stimulation of cell division correlates with the maximum PRL receptor numbers that are present on the Nb2 cells at that time. In addition, PRL has been shown to have a biphasic effect on the expression of the interferon regulatory factor 1 (IRF-1) gene during the cell cycle of Nb2 cells; the first effect occurs in early G₁, and the second in early S phase of the cell cycle (Stevens *et al.* 1995). Interestingly, the second peak of IRF-1 expression (early S phase) requires the continuous presence of PRL during the G₁ phase of the cell cycle. Since IRF-1 expression is closely related to the PRL stimulation of mitogenesis in the Nb2 cells, the maintenance of PRL-receptor numbers during the G₁ phase of the cell cycle may be important for the biphasic stimulation of IRF-1 expression.

Acknowledgement

This research was supported by funds from the Wayne State University School of Medicine.

References

- Ali S, Pellegrini I & Kelly PA 1991 A prolactin-dependent immune cell line (Nb2) expresses a mutant form of the prolactin receptor. *Journal of Biological Chemistry* **266** 20110-20117.
- Buckley AR, Montgomery DW, Kibler R, Putnam CW, Zukoski CF, Gout PW, Beer CT & Russell DH 1986 Prolactin stimulation of ornithine decarboxylase and mitogenesis in Nb2 node lymphoma cells: The role of protein kinase C and calcium mobilization. *Immunopharmacology* **12** 37-51.
- Campbell GS, Argetsinger LS, Ihle JN, Kelly PA, Rillema JA & Carter-Su C 1994 Activation of JAK tyrosine kinase by prolactin receptors in Nb2 cells and mouse mammary gland explants. *Proceedings of the National Academy of Sciences of the USA* **91** 5232-5236.
- Clevenger CV, Ngo W, Sokol DL, Luger SM & Gerwitz AM 1995 VAV is necessary for prolactin-stimulated proliferation and is translocated into the nucleus of a T cell line. *Journal of Biological Chemistry* **27** 13246-13253.
- Clevenger CV, Torigoe T & Reed JC 1994 Prolactin induces rapid phosphorylation and activation of prolactin receptor associated Raf-1 kinase in a T-cell line. *Journal of Biological Chemistry* **269** 5559-5565.
- Erwin RA, Kirken RA, Malabarba MG, Farrar WL & Rui H 1995 Prolactin activates RAS via signaling proteins Shc, growth factor receptor bound 2, and Son of Sevenless. *Endocrinology* **136** 3512-3518.
- Gout PW, Beer CT & Noble RL 1980 Prolactin-stimulated growth of cell cultures established from malignant Nb rat lymphomas. *Cancer Research* **40** 2433-2436.
- Gout PW, Noble RL & Beer CT 1985 Cultured Nb2 rat lymphoma cells in endocrine and cancer research. *Biochemistry and Cell Biology* **64** 659-666.
- Kelly PA, Boutin J, Jolicoeur C, Okamura H, Gagnon J, Ederly M, Sirota M, Banville D, Dusanter-Fourt I & Djiane J 1988 Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family. *Cell* **53** 69-77.
- Murphy PR, DiMatta GE & Friesen HG 1988 Role of calcium in prolactin-stimulated c-myc gene expression and mitogenesis in Nb2 lymphoma cells. *Endocrinology* **122** 2476-2485.
- Noble RL, Beer CT & Gout PW 1980 Evidence *in vivo* and *in vitro* of a role for the pituitary in the growth of malignant lymphomas in Nb rats. *Cancer Research* **40** 2437-2440.
- Rao Y, Buckley DJ & Buckley AR 1995 Rapid activation of mitogen activated protein kinase and p21ras by prolactin and interleukin 2 in rat Nb2 node lymphoma cells. *Cell Growth & Differentiation* **6** 1235-1244.
- Richards JF, Beer CT, Bourgeault C, Chen K & Gout PW 1982 Biochemical response of lymphoma cells to mitogenic stimulation by prolactin. *Molecular and Cellular Endocrinology* **26** 41-49.
- Rillema JA & Lawson DM 1994 Identification of two specific prolactin binding sites on Nb2 rat lymphoma cells. *Proceedings of the Society for Experimental Biology and Medicine* **205** 75-79.
- Rillema JA, Tarrant TM & Linebaugh BE 1989 Studies on the mechanism by which prolactin regulates protein, RNA, and DNA synthesis in Nb2 lymphoma cells. *Biochimica et Biophysica Acta* **1014** 78-82.
- Shiu RPC, Elsholtz HP, Tanaka T, Friesen HG, Gout PW, Beer CT & Noble RL 1983 Receptor-mediated mitogenic action of prolactin in a rat lymphoma cell line. *Endocrinology* **113** 159-165.
- Stevens AM, Wang Y-F, Sieger KA, Lu H-F & Yu-Lee L-Y 1995 Biphasic transcriptional regulation of the interferon regulatory factor-1 gene by prolactin: involvement of γ -interferon-activated sequence and stat-related proteins. *Molecular Endocrinology* **9** 513-525.