Prolactin Receptor and Signal Transduction to Milk Protein Genes (43763)

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Abstract. **After cloning of the mammary gland prolactin (PRL) receptor cDNA, a functional assay was established using co-transfection of PRL receptor cDNA together with a milk protein promoter/chloramphenicol acetyl transferase (CAT) construct in Chinese hamster ovary (CHO) cells. Different mutants of the PRL receptor were tested in this CAT assay to delimit the domains in the receptor necessary for signal transduction to milk protein genes. In CHO cells stably transfected with PRL receptor cDNA, high numbers of PRL receptor are expressed. By metabolic labeling and immunoprecipitation, expressed PRL receptor was identified as a single species of 100 kDa. Using these cells, we analyzed the effects of PRL on intracellular free Ca^{++} concentration. PRL stimulates Ca^{++} entry and induces secondary Ca^{++} mobilization. The entry of Ca^{++} is a result of an increase in K^+ conductance that hyperpolarizes the membranes. We have also analyzed tyrosine phosphorylation induced by PRL. In CHO cells stably transfected with PRL receptor cDNA, PRL induced a very rapid and transient tyrosine phosphorylation of a 100-kDa protein which is most probably the PRL receptor. The same finding was obtained in mammary membranes after PRL injection to lactating rabbits. Whereas tyrosine kinase inhibitors genistein and lavendustin were without effect, PRL stimulation of milk protein gene promoters was partially inhibited by 2 μM herbimycin in CHO cells co-transfected with PRL receptor cDNA and the β lactoglobulin CAT construct. Taken together these observations indicate that the cytoplasmic domain of the PRL receptor interacts with one or several tyrosine kinases, which may represent early postreceptor events necessary for PRL signal transduction to milk protein genes.** [P.S.E.B.M. 1994, Vol 206]

Prolactin (PRL) is known to be involved in a wide range of biological functions including lactation, reproduction, osmoregulation, and immunomodulation (1). The best characterized are those exerted on the mammary gland, where PRL has been shown to stimulate both growth and differentiation as measured by milk protein gene expression. The initial step in PRL action on the mammary cell involves its interaction with a specific membrane receptor which belongs to the recently described cytokine receptor super family (2). The subsequent steps, resulting in the activation of the transcription of specific genes, remain largely unknown. Two forms of the PRL receptor have been cloned in several organs and species. These two receptor forms differ by the size of their cytoplasmic domain which is either long (357 residues), as in rabbit mammary gland (3), or short (57 residues), as in rat liver (4). This review summarizes the results that we recently obtained on the functional domains of the long form of the PRL receptor and on the early postreceptor events in PRL signal transduction.

Functional Domains of the PRL Receptor

A functional assay for PRL receptor was developed several years ago in our laboratory (5). It involves co-transfection in Chinese hamster ovary (CHO) cells of PRL receptor cDNA together with a chimeric gene containing milk protein promoter (β lactoglobulin) fused to chloramphenicol acetyl transferase (CAT) as a reporter gene. Using this functional assay we have shown that only the long form of the PRL receptor is able to stimulate milk protein gene

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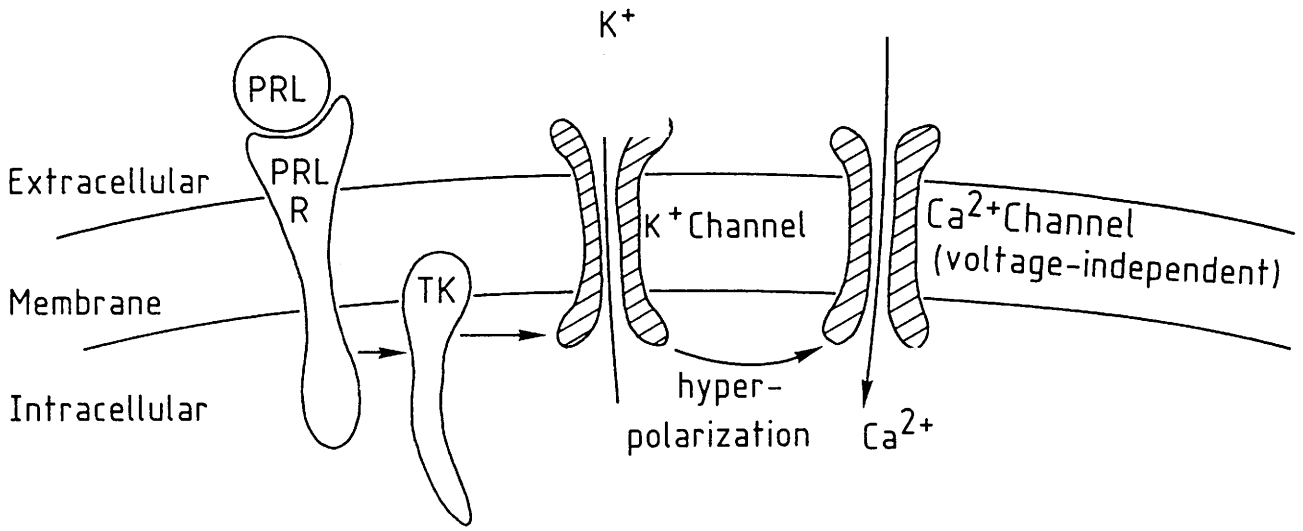

Figure 1. The proposed mechanism for PRL stimulation of Ca^{++} entry in stably transfected CHO cells. After interaction of PRL with its receptor, a potassium (K^+) channel is activated via a tyrosine kinase (TK). The hyperpolarization induced by K^+ exit stimulates Ca^{++} entry via a voltage independent Ca^{++} channel.

promoters (6). It appears that both extracellular and cytoplasmic domains of the PRL receptor participate in signal transduction. Interestingly, while these two domains were inactive when expressed separately, a partial restoration of the activity of the full-length receptor was observed when the two parts of the receptor (extracellular and membrane anchored cytoplasmic domain) were co-expressed in the same CHO cell (7). In the cytoplasmic domain, a limited region (24 residues) proximal to the transmembrane domain appears critical for signal transduction (8). This domain is rich in proline and contains a box of eight amino acids which is also found in several other members of the cytokine receptor family.

Early Events in PRL Signal Transduction

The stimulation of milk protein gene transcription is a long-term effect in PRL signal transduction occurring over several hours. To study early events, we developed CHO cells stably transfected with PRL receptor cDNA and used these cells to analyze several early postreceptor events.

Effect of PRL on Intracellular Ca^{++} Concentrations. We examined the effects of PRL on intracellular free Ca^{++} concentrations in individual CHO cells over-expressing functional PRL receptors. $[Ca^{++}]i$ was determined by dual emission microspectrofluorimetry using Indo 1 as a Ca^{++} fluorescent probe. We demonstrated (9) that exposure of transfected cells to physiological concentrations of PRL (100 ng/ml) for 15 sec resulted in an increase in $[Ca^{++}]i$ in the majority (67%) of cells, providing these had been deprived of serum for at least 6 hr. We observed several types of response to PRL, but in all cases the response was delayed, the increase in $[Ca^{++}]i$ starting from 15 sec to 120 sec after hormone addition. Moreover, PRL responses differed in their kinetics. In 32% of responsive cells, the PRL-induced increase in $[Ca^{++}]i$ was very slow and of low amplitude; the maximal value was reached at 1–2 min, after which $[Ca^{++}]i$ returned slowly to basal values 5 to 7 min after hormone addition. If the experiments were performed in a Ca^{++}-free medium this type of response was completely abolished, suggesting that this slow increase in $[Ca^{++}]i$ was due to a PRL-stimulated Ca^{++} entry. A second type of kinetic in the response was observed in 35% of the cells where maximal $[Ca^{++}]$ increase was reached more rapidly and the return to basal value was also more rapid. This second type of response appeared to be the result of a delayed Ca^{++} mobilization from internal stores. This phenomenon is partially dependent of Ca^{++} entry suggesting that Ca^{++} entry may induce Ca^{++} mobilization.

Electrophysiological techniques were used to better characterize the early effects of PRL on membrane ion conductance. We observed recently (Prevarskaya *et al.*, submitted[2]) that application of PRL to the transfected CHO cells increased voltage-dependent potassium (K^+) conductance. Subsequent addition of 30-n*M* charybdotoxin (a specific blocker of K^+ channel) reduced K^+ currents to control levels and completely blocked the effects of PRL on $[Ca^{++}]i$ increase and membrane potential. Cell-free patch-clamp experiments showed that PRL directly stimulates K^+ channel activity. Adenosine triphosphate (ATP), a protein

[2] Prevarskaya N, Skryma R, Vacher P, Daniel N, Bignon C, Djiane J, Dufy B. Characterization of early effects of prolactin on membrane ion conductance in CHO cells stably transfected with PRL receptor cDNA. Am J Physiol (submitted 1994).

kinase substrate, increased the channel open probability. These results suggest the existence of a regulatory complex involving a protein kinase tightly associated with a PRL-regulated K^+ channel in CHO cells. As depicted schematically (Fig. 1), we conclude that PRL stimulates voltage-dependent charybdotoxin-sensitive K^+ channels by activation of a protein kinase which probably associates with the PRL receptor. The resulting hyperpolarization stimulates Ca^{++} entry, apparently through voltage-insensitive Ca^{++} channels.

Effect of PRL on Tyrosine Phosphorylation. Recently a number of laboratories presented evidence that tyrosine kinase activation constitutes an early event after ligand binding to several members of the cytokine receptor family including growth hormone (GH) (10) and PRL (11). We first tested CHO cells stably transfected with PRL receptor cDNA to find whether PRL induced tyrosine phosphorylation of proteins associated with its receptor. Solubilized proteins from CHO transfectants, both treated and not treated with PRL, were immunoprecipitated using polyclonal anti-PRL receptor antibodies and then analyzed by Western blot with antiphosphotyrosine antibodies. We observed that PRL very rapidly induced (1 min) tyrosine phosphorylation of a protein of molecular ratio 100,000. This phosphorylation was transient, being undetectable 10 min after addition of physiological concentrations of PRL (Fig. 2). We tested whether this tyrosine phosphorylated protein was in fact the PRL receptor by reprobing the same blot with anti-PRL receptor antibodies. PRL receptor was detected as a doublet very close to the position of the tyrosine phosphorylated protein (Fig. 2). This result is consistent with the possibility that the phosphorylated band is the PRL receptor itself migrating a little slower when phosphorylated. It is of course possible that a protein distinct from the PRL receptor, but tightly associated with it and having the same molecular weight, was also phosphorylated.

To determine if the same phenomenon occurs in the mammary gland, we analyzed tyrosine phosphorylation in mammary membranes after PRL injection to lactating rabbits. As shown in Fig. 3, PRL receptors which had been immunoprecipitated by a monoclonal

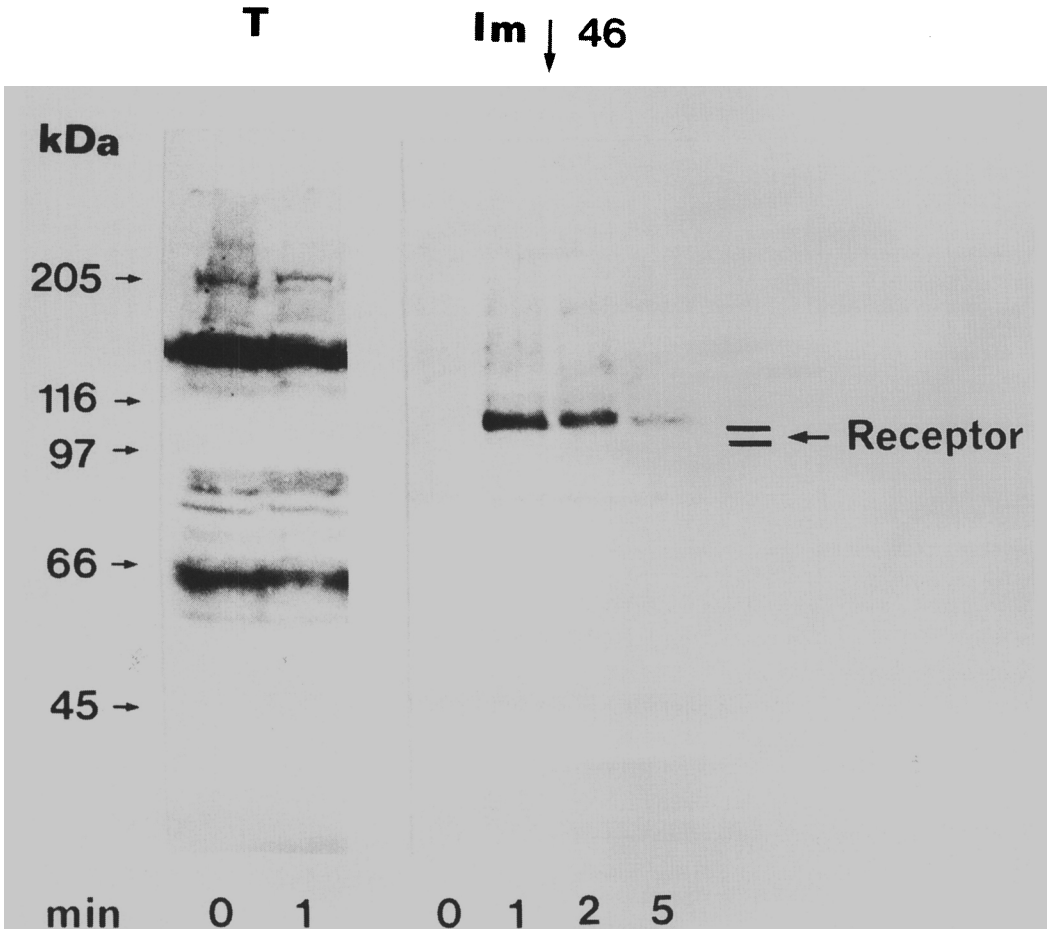

Figure 2. Antiphosphotyrosine immunoblot of solubilized proteins from CHO cells stably transfected with PRL receptor cDNA. Cells were incubated with 400 ng/ml of ovine PRL (oPRL) for the indicated time. Cells were lysed, and solubilized proteins were subjected directly (T), or after immunoprecipitation, with polyclonal antibodies to the PRL receptor (Im 46), to Western blotting analysis using antiphosphotyrosine antibodies (α PY-4G10). Molecular weights ($\times 10^{-3}$) of the protein standards are indicated. The same blot was reprobed with anti-PRL receptor antibodies and the receptor was detected as a doublet depicted in the figure (= Receptor).

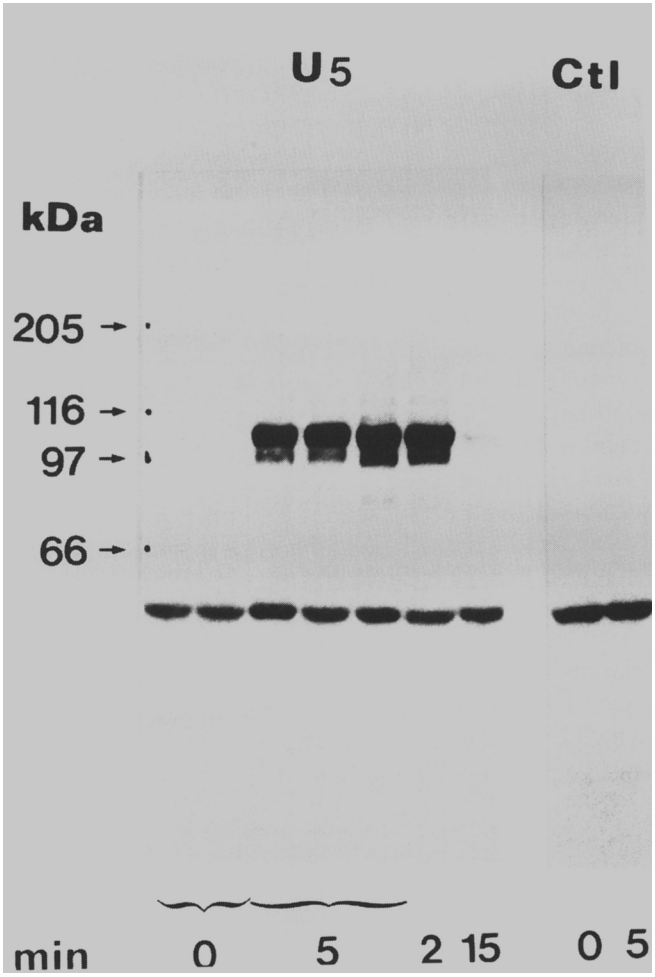

Figure 3. Antiphosphotyrosine immunoblot illustrating the time course of PRL promoted tyrosyl phosphorylation of its own receptor in the mammary gland. Lactating rabbits at 15 days of lactation were pretreated with bromocriptine (2 mg/day) for 36 hr to decrease endogenous PRL levels before intravenous injection of oPRL (1 mg) or saline (O). The animals were sacrificed 2 min, 5 min, or 15 min after PRL injection. The mammary glands were rapidly frozen in liquid nitrogen and stored at −80°C. Microsomal membranes were prepared in the presence of several antiprotease inhibitors and solubilized in 1% Triton X100. Monoclonal antibody to the prolactin receptor (U5) or control antibody (Ctl) was used for immunoprecipitation, and the immunoprecipitated proteins were subjected to Western blot using antiphosphotyrosine antibody (α PY-4G10 UB1). Molecular weight of the protein standards used are indicated (kDa).

antibody to the PRL receptor (U5) were recognized on a Western blot by antiphosphotyrosine antibodies, but only if the animal was previously treated with PRL. The phosphorylation of the receptor occurred very rapidly (2 min after PRL injection) and was transient since phosphorylation dramatically decreased 15 min after PRL injection. We did not detect associated phosphorylated protein of higher molecular weight (120–130 kDa) as shown for the GH receptor in several cell lines (12) and for PRL receptor in Nb2 cell line (10) where PRL exerts a proliferative effect. This protein was recently identified as Janus kinase 2 (JAK2) (13). It remains possible that this kinase dissociated during preparation of the mammary membranes or that it is only weakly active in highly differentiated mammary cells during lactation.

Effects of Protein Kinase Inhibitors on PRL Stimulation of Milk Protein Promoters

Our functional test for the PRL receptor described earlier was used to analyze the effects of different protein kinase inhibitors on PRL stimulation of milk protein promoters. Because of the toxicity associated with many protein kinase inhibitors, we have developed a treatment protocol which restricts the time of exposure with these inhibitors to 4 hr during the critical period for signal transduction. Only 2 hr of contact with PRL are sufficient to obtain a significant effect on the milk protein promoter assayed 24 hr later. Several

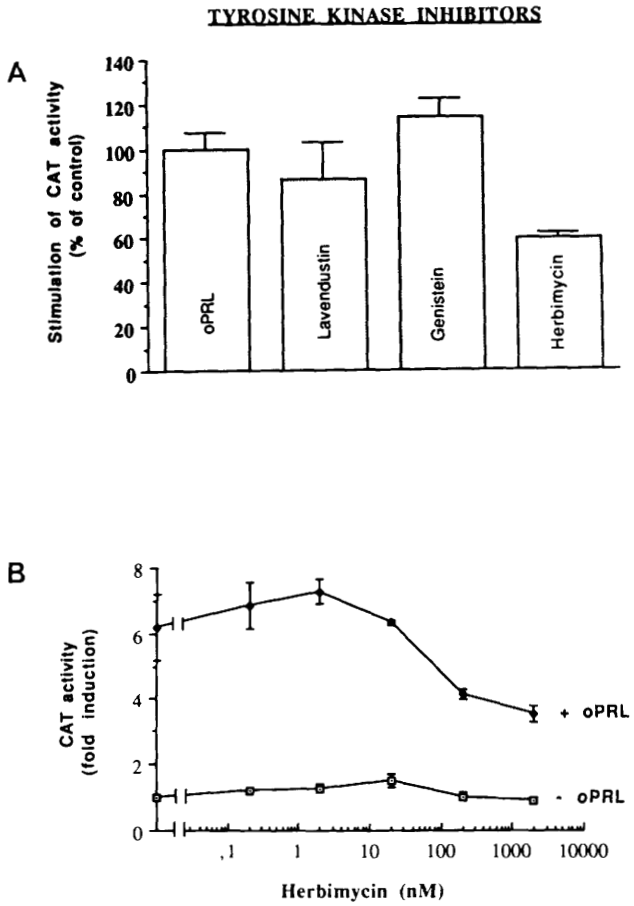

Figure 4. Effect of different tyrosine kinase inhibitors on the stimulation of chloramphenicol acetyl transferase (CAT) activity induced by PRL in CHO cells co-transfected with PRL receptor cDNA and β lactoglobulin promoter/CAT construct. Prolactin was added to the culture medium of co-transfected CHO cells for 2 hr. The inhibitors were added 2 hr before PRL and maintained for two additional hours during PRL incubation. After this time the culture medium was changed and after 24 hr incubation of the CAT activity in cell homogenates was determined. (A), Prolactin alone (oPRL, 400 ng/ml) or oPRL with the various inhibitors (concentrations in parentheses) were added to the culture medium: lavendustin (50 μg/ml), genistein (50 μg/ml), herbimycin (2 μ*M*). (B), Dose response curve for the inhibitory effect on CAT activity resulting from herbimycin incubation with co-transfected CHO cells plus or minus PRL.

tyrosine kinase inhibitors were tested and, as shown in Fig. 4, lavendustin A (50 μg/ml) and genistein (50 μg/ml) were without effect. By contrast, we observed a dose dependent inhibition of PRL-induced CAT activity with herbimycin. When this compound was added to the culture medium at 200 μ*M*, 2 hr before PRL (400 ng/ml) and during two additional hours in association with PRL, an inhibition of 50% of the PRL effect on CAT activity was observed. This result suggests that a tyrosine kinase sensitive to herbimycin is probably required for PRL signal transduction to milk protein genes. To confirm this hypothesis we analyzed the effects of okadaic acid, a protein phosphatase inhibitor. This compound, which is known to inhibit phosphatase 1 and 2A when used at 200 n*M*, exhibited a PRL-like effect on β lactoglobulin promoter since we observed a 2-fold increase in CAT activity in CHO cells co-transfected with PRL receptor cDNA and the β lactoglobulin CAT construct. Addition of PRL to these cells further increased CAT activity to 4-fold compared with control. These results are in agreement with a recent report (14) indicating that phosphate inhibitors mimic PRL effects in mammary gland explants.

Summary and Conclusions

Taken together, the results summarized in this review are consistent with the conclusion that a specific region of the cytoplasmic domain of the PRL receptor interacts with one or several tyrosine kinases which phosphorylate the PRL receptor itself, and other substrates like ion channels (K^+ channel) or transcriptional factors. The activation of tyrosine kinase may be a key event in the transduction of the PRL signal to milk protein genes. The tyrosine kinase involved remains to be identified, but it appears to be sensitive to herbimycin. The likely involvement of the newly identified tyrosine kinases of the Janus kinase (JAK) family (JAK1, JAK2, tyk2) is currently under investigation in our laboratory.

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