Effect of hormones on dissociation of prolactin from the rabbit mammary gland prolactin receptor

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Using microsomes prepared from rabbit mammary gland, the dissociation of prolactin (PRL) from its receptor was determined in the presence of peptide hormones or various concentrations of PRL. Among the hormones tested, PRL (ovine, mouse and bullfrog), human growth hormone and human placental lactogen each accelerated the dissociation of PRL in a manner proportional to their receptor-binding activities. Hormone-dependent dissociation was observed at higher concentrations than those at which the binding of PRL was completely inhibited by lactogenic hormones. In the concentration range 0.1 ng/ml–10 µg/ml, PRL increased the rate of dissociation in a logarithmic concentration-dependent manner. It was concluded that the dissociation of PRL from its receptor caused by lactogenic hormones is dependent on the hormone concentration. Arrhenius plot analysis revealed that PRL changed the frequency factor for the dissociation reaction. PRL in the medium inhibited the re-association of dissociated PRL. The data also suggested that PRL regulates the rate of dissociation by interacting with the PRL–receptor complex.

INTRODUCTION

Prolactin (PRL) induces its hormonal message through the action of binding to its membrane receptor in the target organ. The reaction of PRL with its receptor is a reversible and bimolecular reaction, but the dissociation of PRL has received little attention.

The interaction of PRL with its receptor has been evaluated by thermodynamic analysis (Haro & Talamantes, 1985; Sakai *et al.*, 1990). We showed that PRL forms a very stable complex with the rabbit mammary gland PRL receptor (Sakai *et al.*, 1990), and the rate of PRL dissociation from its receptor is low (Kelly *et al.*, 1980). However, it is also known that the presence of PRL during the dissociation reaction increases the rate of PRL dissociation above that which is observed in its absence (Perry & Jacobs, 1978; Shiu, 1979; Haro & Talamantes, 1986). This mechanism of PRL action is not yet understood, but the following possibilities are conceivable: (a) PRL is available to readily re-associate with the receptor immediately after dissociation, and/or (b) PRL alters the rate of dissociation of PRL from its receptor by interacting with the PRL–receptor complex. These hypotheses are examined in the present investigation by thermodynamic analysis and the use of a binding assay. In the present experiments, after ^{125}I -PRL was allowed to associate with its microsomal receptor, the rate constant of dissociation of ^{125}I -PRL from the receptor (k_{-1}) was determined in the presence of various concentrations of PRL. The effects of PRL on the change in k_{-1} for ^{125}I -PRL were evaluated by Arrhenius plotting and thermodynamic analysis.

It is well known that other lactogenic hormones, such as human growth hormone (hGH) and placental lactogen (PL), can bind to the PRL receptor in the mammary gland. Until recently the effects of these hormones on the dissociation of PRL from its receptor were unknown. Using various peptide hormones, the specificity of binding to the PRL receptor is also examined in the present paper.

MATERIALS AND METHODS

Hormones

Ovine PRL (NIADDK-oPRL-17; 30 units/mg), human GH (NIADDK-hGH-01; 1.7 units/mg), oGH (NIADDK-oGH-15, 1.9 units/mg), ovine follicle-stimulating hormone (NIADDK-oFSH-14) and ovine luteinizing hormone (NIADDK-oLH-24) were kindly supplied by NIADDK, Bethesda, MD, U.S.A. Bullfrog (bu) and mouse (m) PRL were gifts from Dr. K. Yamamoto, Waseda University, Waseda, Japan, and Professor K. Kohmoto, University of Tokyo, Tokyo, Japan, respectively (Yamamoto & Kikuyama, 1982; Kohmoto *et al.*, 1984). Insulin was a gift from Novo Industri A/S, Bagsvaerd, Denmark. hPL (i020) was obtained from UCB Bioproducts, Brussels, Belgium. Bovine thyroid-stimulating hormone (bTSH; T-2026) was obtained from Sigma, St. Louis, MO, U.S.A.

Association and dissociation procedures

PRL was iodinated using lactoperoxidase and H_2O_2 (Sakai *et al.*, 1975), and the specific radioactivity of ^{125}I -PRL was about 3.2 MBq/µg. Microsomes were prepared from mid-lactating rabbit mammary glands. The protocol for the PRL-binding assay has been described previously (Sakai *et al.*, 1990). In the present investigation the concentrations of microsomes and ^{125}I -PRL employed were doubled compared with those in Sakai *et al.* (1990) in order to obtain sufficient radioactivity for the analysis. The assay buffer consisted of 25 mM-Tris/10 mM-MgCl₂/1 mM-phenylmethanesulphonyl fluoride/0.02 % Na₂S₂O₃/0.2 % BSA, and was adjusted to pH 7.6 with HCl at the temperature at which it was used. Microsomes (150 µg of protein) were incubated for 16 h at 20 °C with ^{125}I -PRL (60 000–70 000 c.p.m.) in the presence or absence of unlabelled PRL (1 µg). At the end of incubation, the reaction mixture was diluted with 3 ml of assay buffer and centrifuged at 2300 g for 15 min at 20 °C, and the supernatant was discarded. Subsequently, 1 ml of assay buffer containing unlabelled peptide hormones was added to each tube. The tubes

Abbreviations used: PRL, prolactin; PL, placental lactogen; GH, growth hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone; the prefixes o, h, b, m and bu refer to the ovine, human, bovine, mouse and bullfrog hormones respectively; k_{-1} , dissociation rate constant; A , frequency factor; E_a , activation energy; $\Delta G^\ddagger$, free energy of activation; $\Delta H^\ddagger$, enthalpy of activation; $\Delta S^\ddagger$, entropy of activation.

were further incubated at various temperatures, and the temperature was maintained within $\pm 0.1^\circ\text{C}$ during dissociation. Upon termination of the dissociation reaction, the reaction mixture was diluted with 3 ml of assay buffer and the tubes were processed as described above. Radioactivity in the tubes was measured in a γ -radiation counter. The value for k_{-1} was calculated from the slope of the dissociation curve. Experiments were performed in triplicate, and each determination was repeated three times.

Thermodynamic analysis

The activation energy (E_a) and the frequency factor (A) for dissociation were calculated by the Arrhenius equation:

$$\log k_{-1} = \log A - (E_a/2.303 R) (1/T)$$

where R and T are the gas constant and the absolute temperature respectively. The free energy of activation ($\Delta G^\ddagger$), enthalpy of activation ($\Delta H^\ddagger$) and entropy of activation ($\Delta S^\ddagger$) for dissociation were calculated according to transition state theory (Sakai *et al.*, 1990). All thermodynamic data were standardized at 25°C .

Statistical analysis

A straight line was constructed using the least-squares linear-regression method. In the present investigation, correlation coefficients were larger and smaller than 0.95 and -0.95 respectively. Statistical analysis was performed using Student's two-tailed t test. Differences were considered to be significant at $P < 0.05$.

RESULTS

Effects of hormones on association and dissociation of ^{125}I -PRL at 25°C

The binding of ^{125}I -PRL to its receptor was determined in the presence of various concentrations of peptide hormones. As

Fig. 1. Competitive displacement by peptide hormones of ^{125}I -PRL binding

Incubation was carried out for 24 h at 25°C in a total volume of 1 ml. Data are expressed as percentage binding, calculated from specific binding of ^{125}I -PRL in the presence of competitor hormones divided by that in the absence of competitor hormone. The symbol ■ represents results with oFSH, oLH, bTSH and insulin. The results are means of three determinations.

shown in Fig. 1, the binding of ^{125}I -PRL was inhibited by the presence of hGH, oPRL, hPL or mPRL. The dose-response curves were parallel. hGH was the most effective of the hormones examined. At 30 ng of hGH/ml, more than 98 % of specific ^{125}I -PRL binding was suppressed. High concentrations of buPRL inhibited slightly the binding of ^{125}I -PRL. Non-lactogenic hormones (oFSH, oLH, bTSH, oGH and insulin) showed almost no inhibitory action.

Various hormones were tested during the 24 h dissociation reaction, and the data were compared with data obtained in the absence of hormone (Table 1). Lactogenic hormones at $1\ \mu\text{g/ml}$ increased the rate of dissociation of ^{125}I -PRL from its receptor with the order $\text{hGH} > \text{oPRL} > \text{hPL} > \text{mPRL} > \text{buPRL}$. This hierarchy was the same as that of hormone concentrations necessary to inhibit PRL binding by 50 % (IC_{50}), and the relationship between binding (%) and inhibitory potency could be expressed as:

$$\text{binding (\%)} = 0.154 \times \log \text{IC}_{50} - 11.2$$

Although the binding of ^{125}I -PRL was completely inhibited by hGH or oPRL at 100 ng/ml, the amount of ^{125}I -PRL dissociated was significantly less than that at $1\ \mu\text{g/ml}$. Non-lactogenic hormones did not change the dissociation of ^{125}I -PRL.

Using low concentrations of ^{125}I -PRL, the binding of ^{125}I -PRL to its receptor was determined in order to estimate the amount of re-association of PRL (Fig. 2). It has been reported that both PRL and its receptor after dissociation remain active for re-association (Powell & Hollander, 1978; Van Der Gugten *et al.*, 1980). The amount of specific binding, obtained in the absence of unlabelled PRL, increased linearly with increasing concentrations of ^{125}I -PRL. About 31 % of total ^{125}I -PRL could participate in binding to the receptor. At 0 h of dissociation, the amount of specific binding of ^{125}I -PRL was about 19000 c.p.m./tube under standard binding assay conditions. If the bound PRL has dissociated completely, it is reasonably expected that maximum binding is less than 6000 c.p.m. during the 24 h association period. At the end of the 24 h dissociation reaction, 8580 ± 374 c.p.m. (mean $\pm$ s.d.) had dissociated from the receptor in the presence of $10\ \mu\text{g}$ of unlabelled PRL/ml (Fig. 3). The inclusion of unlabelled PRL inhibited the binding of ^{125}I -PRL to its receptor. The data indicated that, at 100 ng of unlabelled PRL/ml, the binding of ^{125}I -PRL is negligible.

Effect of PRL on dissociation of ^{125}I -PRL

The effects of PRL concentration and temperature on the dissociation of ^{125}I -PRL from the receptor were examined. The concentration of PRL and the incubation temperature were varied in the ranges 0.1–10000 ng/ml and 13 – 37°C respectively. Values at 24 h of dissociation were compared with those at 0 h of dissociation. As shown in Fig. 3(a), dissociation was a very slow reaction in the absence of PRL; however, the inclusion of PRL increased the rate of dissociation. There was a linear relationship between dissociation and temperature, and the rate of dissociation was increased when the incubation was carried out at higher temperatures. The slope of the regression line was clearly changed in a concentration-dependent manner. However, all regression lines crossed the 'temperature' axis at almost the same position ($14.6 \pm 0.7^\circ\text{C}$), suggesting that dissociation virtually does not occur at temperatures lower than 14.6°C . At 13°C , no dissociation was observed after 24 h. At lower temperatures, dissociation of PRL has been reported to be negligible (Shiu & Friesen, 1974; Haro & Talamantes, 1985). In Fig. 3(b) the same data are re-plotted against the PRL concentration in order to estimate the minimum concentration of PRL necessary for dissociation. It can be estimated that dissociation is negligible at concentrations lower than $1.5 \pm 0.8\ \mu\text{g}$ of PRL/ml. This plotting

Table 1. Effect of peptide hormones on the dissociation of ¹²⁵I-PRL from its receptor

Dissociation was carried out for 24 h at 25 °C. The 100 % value was obtained in the absence of hormone. The inhibitory potency (IC₅₀) of each hormone was calculated from the data in Fig. 1 (mean ± s.d., n = 3). N.D., not determined.

Hormone (1 µg)	Binding (%)	IC ₅₀ (ng/ml)
hGH	74.1 ± 1.6 (82.9 ± 1.2)*	1.3 ± 0.2
oPRL	76.2 ± 1.4 (89.9 ± 4.1)*	2.2 ± 0.3
hPL	83.1 ± 2.3	51.0 ± 5.2
mPRL	88.5 ± 1.0	260 ± 23
buPRL	96.5 ± 0.8	3000 ± 300†
oGH, oFSH, oLH, bTSH, insulin	100.0	N.D.

* The assay was carried out in the presence of 100 ng of hGH or oPRL.
† Calculated on the assumption that the dose-response curve of buPRL is parallel to other curves.

Fig. 2. Effect of PRL concentration on the binding of ¹²⁵I-PRL to its receptor

Incubation was carried out at 25 °C for 24 h in the presence of 0, 0.1 or 1 µg of unlabelled PRL in a total volume of 1 ml. Values obtained in the presence of 1 µg of PRL have been subtracted; ●, 0 ng; ○, 0.1 µg of PRL.

analysis allowed me to predict not only the concentration- and temperature-dependency of the dissociation reaction but also the minimum PRL concentration and temperature necessary for dissociation.

Thermodynamic analysis of the dissociation reaction

The dissociation reaction was carried out in the presence of various concentrations of unlabelled PRL (1–1000 ng/ml) and at 19, 24 and 29 °C in order to evaluate thermodynamically the effects of PRL and temperature on the change in *k*₋₁. The dissociation of ¹²⁵I-PRL from its receptor changed in a time- and concentration-dependent manner. Rapid dissociation, observed during the first 4 h incubation period (Kelly *et al.*, 1980; Sakai

Fig. 3. Plots of the rate of ¹²⁵I-PRL dissociation against temperature (a) and PRL concentration (b)

Curves are labelled with numbers indicating (a) log{[PRL](ng/ml)} and (b) temperature (°C). The curve indicated as (0) in (a) indicates in the absence of PRL (dilution only). Values at 24 h of dissociation (*A*₂₄) were compared with those at 0 h of dissociation (*A*₀). The dissociation rate is expressed as 1/24 × ln(*A*₂₄/*A*₀). Data are the means of three determinations, and the s.d. values were within 5 % of the mean.

Fig. 4. Arrhenius plot analysis of the dissociation reaction between PRL and its receptor

The dissociation reaction was carried out in the presence of various concentrations of unlabelled PRL (log{[PRL](ng)}) as indicated. The value (0) indicates the absence of PRL. Values obtained in 0, 4, 9, 16 and 24 h of dissociation were used in the calculation of *k*₋₁. The mean value of three determinations was plotted as a function of temperature (1/*T*).

et al., 1990), could be minimized by temperatures lower than 29 °C. It was confirmed that the dissociation reaction of ¹²⁵I-PRL obeyed first-order kinetics in both the absence and the presence of PRL during dissociation. The value of *k*₋₁ changed as a function of incubation temperature. Fig. 4 shows the Arrhenius plots obtained in the presence of various concentrations of PRL.

Table 2. Values for the free energy ($\Delta G^\ddagger$), enthalpy ($\Delta H^\ddagger$) and entropy ($\Delta S^\ddagger$) of activation and activation energy (E_a) for dissociation of PRL from its receptor

Results were obtained in the presence of various concentrations of PRL at 25 °C and are means $\pm$ S.D. ($n = 3$). * $P < 0.05$, ** $P < 0.01$ compared with 0 ng control.

PRL (ng/ml)	$\Delta G^\ddagger$ (kJ·mol ⁻¹)	$\Delta H^\ddagger$ (kJ·mol ⁻¹)	$\Delta S^\ddagger$ (J·mol ⁻¹ ·K ⁻¹)	E_a (kJ·mol ⁻¹)
0	105.6 $\pm$ 0.2	53.6 $\pm$ 2.0	-174.8 $\pm$ 6.0	56.1 $\pm$ 2.0
1	104.9 $\pm$ 0.2	54.6 $\pm$ 0.6	-166.6 $\pm$ 3.4	57.1 $\pm$ 0.6
10	104.0 $\pm$ 0.1**	56.2 $\pm$ 1.5	-160.4 $\pm$ 5.0	58.7 $\pm$ 1.5
100	103.7 $\pm$ 0.2**	57.5 $\pm$ 0.2	-155.0 $\pm$ 1.0*	60.0 $\pm$ 0.2
1000	103.4 $\pm$ 0.1**	58.3 $\pm$ 1.2	-148.5 $\pm$ 1.6*	60.8 $\pm$ 1.2

Fig. 5. PRL-dependent changes in A (●) and k_{-1} (○) at 25 °C

The value for k_{-1} was calculated from the Arrhenius plot shown in Fig. 4 by interpolation. Vertical bars represent the S.D. ($n = 3$). * $P < 0.05$, ** $P < 0.01$ compared with the 0 ng control.

A plot of $\log k_{-1}$ against $1/T$ generated a straight regression line, and the slopes of these plot lines were nearly equivalent. This parallelism indicated that PRL changed the A value of the dissociation reaction rather than the E_a for dissociation. The values of k_{-1} and A were greatly changed, depending on the concentration of PRL. At 1000 ng of PRL/ml, the values for A and k_{-1} were about 17.0-fold and 2.8-fold respectively greater than in the 0 ng control ($P < 0.01$), and the E_a was increased from 56.1 to 60.8 kJ·mol⁻¹ (not significant).

Using the data for E_a and k_{-1} , the dissociation reaction was analysed by thermodynamic analysis (Table 2). The positive sign of $\Delta H^\ddagger$ indicated that the dissociation reaction was endothermic. The values of $\Delta G^\ddagger$ and $\Delta S^\ddagger$ were changed by PRL in a concentration-dependent manner. Differences between the 0 ng control and the experimental groups were very small. The small changes in E_a and $\Delta G^\ddagger$ indicate that PRL influenced the dissociation reaction thermodynamically very little.

Using the data standardized at 25 °C, the effects of unlabelled PRL on changes in A and k_{-1} were analysed. In Fig. 5, values for A (h⁻¹) and k_{-1} (h⁻¹) are plotted against the concentration of PRL (ng/ml). The relationships obtained were

$$\log A = 0.34 \times \log [\text{PRL}] + 8.00$$

and

$$k_{-1} = (2.93 \times \log [\text{PRL}] + 9.12) \times 10^3$$

From the latter equation, it can be predicted that dissociation does not occur at concentrations lower than 0.8 pg of PRL/ml. There was a logarithmic and linear relationship between A and k_{-1} :

$$k_{-1} (\text{h}^{-1}) = 0.009 \times \log A - 0.055$$

DISCUSSION

The inclusion of PRL in the reaction mixture increased the rate of dissociation of PRL from its receptor, and I was able to confirm the results of earlier investigations (Shiu & Friesen, 1974; Perry & Jacobs, 1978; Shiu, 1979; Kelly *et al.*, 1980, 1983; Haro & Talamantes, 1986). As both PRL and the receptor remain active after dissociation for re-association (Powell & Hollander, 1978; Van Der Gugten *et al.*, 1980), it is certain that PRL can interrupt the re-association of dissociated PRL. However, PRL-dependent dissociation was observed at concentrations higher than that at which the binding of ¹²⁵I-PRL was completely inhibited by PRL. The present data further show that other lactogenic hormones, i.e. hGH and hPL, are able to regulate the rate of PRL dissociation from its receptor. The dissociation reaction is endothermic and temperature-dependent. Our previous results suggested that the PRL–receptor complex is further stabilized by the contributions of hydrogen bonds and probably van der Waals contacts after the hydrophobic interaction (Sakai & Suzuki, 1988; Sakai *et al.*, 1990). It is expected that the PRL–receptor complex becomes less stable as the incubation temperature is increased.

At any PRL concentration, the Arrhenius plots generated a straight regression line. The value for the E_a for dissociation was increased by PRL, but this difference was negligible compared with the 0 ng control. The value of A was changed in a manner inversely related to the concentration of PRL. The value of $\Delta S^\ddagger$ increased as the PRL concentration was increased. An increase in A was due largely to the PRL-dependent increase in $\Delta S^\ddagger$. These thermodynamic parameters suggest that, in order for the reaction to occur, PRL must collide with the PRL–receptor complex.

Lactogenic hormones change the rate of dissociation to an extent proportional to their receptor-binding activity. It is evident that PRL at 10 µg/ml can accelerate the dissociation of bound PRL. This action cannot be explained by the mechanistic collision of lactogenic hormones with the PRL–receptor complex, since non-lactogenic hormones had no effects on the association and dissociation of PRL. It is unclear whether the PRL–receptor complex is able to associate with lactogenic hormones. I speculate that the mechanism of hormone action is as follows. Lactogenic hormones are able to interact with the PRL–receptor complex even if the binding affinity is low. This interaction of lactogenic hormones with the PRL–receptor complex may change the receptor-binding properties of PRL. Hormone-dependent dissociation has not been thoroughly examined. However, the reaction probably occurs by a mechanism common to other hormone–receptor interactions, as it is known in general that the inclusion of a particular hormone accelerates the rate of dissociation of that hormone from its receptor. Hormone-dependent dissociation has been reported in the FSH receptor (Cheng, 1975), insulin receptor (Harrison *et al.*, 1978; Posner *et al.*, 1978; Waelbroeck *et al.*, 1979; Kosmakos & Roth, 1980; Gu *et al.*, 1988; Wang *et al.*, 1988; Shymko *et al.*, 1989) and TSH receptor (Amir *et al.*, 1973; Christopher *et al.*, 1980).

The action of PRL on lactation is of much physiological importance: without mother's milk, most mammalian young would not be able to survive. Increases in the number of

mammary gland PRL receptors after parturition have been shown in dairy cattle (Emane *et al.*, 1986; Kazmer *et al.*, 1986), rabbits (Djiane *et al.*, 1977; Grisson & Littleton, 1988), rats (Holcomb *et al.*, 1976; Bohnet *et al.*, 1977; Hayden *et al.*, 1979) and mice (Frantz *et al.*, 1974; Sakai *et al.*, 1978). In rabbits, serum PRL increases at parturition and reaches maximum levels during early lactation (Durand & Djiane, 1977). Both changes undoubtedly increase the concentration of PRL in the mammary gland. An additional merit of this dissociation reaction is that the mammary gland is able to retain PRL in the receptor for a relatively long time period under conditions where the concentration of serum PRL abruptly decreases. All of the above are advantageous in promoting the continuance of lactation by the mammary gland.

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