Mechanism of Biphasic Action of Mono- and Bivalent Salts on the Binding of Prolactin to the Receptor in the Rabbits Mammary Gland

SENKITI SAKAI AND KAORU KHOMOTO

Department of Animal Breeding, Faculty of Agriculture, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan

Abstract

The influence of NaCl, KCl, CaCl₂, and MgCl₂ on the binding of prolactin (PRL) to its receptor was investigated. The salts were dissolved in a metallic ion-free binding buffer and had biphasic effects on changes in the association rate constant (k_{+1}) of PRL binding, depending on their concentrations: there was an increase in the k_{+1} at lower concentrations and a decrease at higher concentrations. The dissociation rate of bound PRL was unaffected. NaCl at any concentration did not change the binding capacity. Bivalent salts, at higher than 25 mM, increased the capacity about 1.6-fold as compared to the 0 mM control. By cross-linking the PRL-receptor complex, the band of a molecular weight (M_r) 34,500 receptor could always be detected on the autoradiogram. An M_r 78,000 receptor appeared only after incubation with bivalent salts. Data indicate that the binding of PRL to an M_r 78,000 receptor is directly regulated by bivalent cation.

The mammary gland has a specific receptor for prolactin (PRL). The binding of PRL to its receptor is a chemical reaction, and thus the importance of the effect of ions and their ionic strength on the binding of PRL to the receptor has been noted (Shiu and Friesen, 1974; Haro and Talamantes, 1985). However, the effects of ions and their ionic strength on the association and dissociation of PRL have not been thoroughly studied. In this study, the assay was carried out in the presence of increasing concentrations of mono- or

bivalent salts. The association rate constant (k_{+1}) and dissociation rate constant (k_{-1}) of PRL were determined in order to clarify the mechanism of the action of salts on stimulation of PRL binding. In the preliminary experiments, the introduction of NaCl or KCl failed to produce maximal stimulation, which was achieved by adding CaCl₂ or MgCl₂ at 25 mM. Recently, we reported that the rabbit mammary gland contains PRL-binding species with a different molecular weight (M_r) (Sakai *et al.*, 1987; Murakami *et al.*, 1988). Under different ionic environments, the species of PRL-binding receptor was also analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

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To whom correspondence should be addressed:
Dr. SENKITI SAKAI
(Tel. 03-812-2111, Ext. 5382)

Materials and Methods

PRL binding assay

Ovine PRL (NIADDK-oPRL-18, 31 IU/mg) was kindly supplied by NIADDK, Bethesda, Md. PRL was iodinated by lactoperoxidase and H_2O_2 , and its specific activity was about 2.775 MBq/ μ g (Sakai *et al.*, 1975). Mammary microsomes obtained from mid-lactating New Zealand White rabbits were used. The procedures for the PRL binding assay, the Scatchard analysis, and the time-course kinetics will be described elsewhere (Sakaki and Suzuki, 1989). In the present experiments, the binding buffer was metallic ion-free, and consisted of 25 mM Tris/HCl (pH 7.4)-1mM phenylmethylsulfonyl fluoride-0.2% bovine serum albumin. NaCl, KCl, $MgCl_2$, and $CaCl_2$ were dissolved in the binding buffer and the pH was adjusted to 7.4. The final concentrations are shown in the text. The microsomes (100 μ g) were incubated at 20°C for 20 h with 40,000 cpm of [125 I]PRL in the presence and absence of excess unlabeled PRL (1 μ g) in a reaction volume of 0.5 ml. When the incubation was terminated, 4 ml of the binding buffer containing 10 mM $MgCl_2$ was added and the tubes were centrifuged at $2,300 \times g$ for 10 min at 4°C. For the Scatchard analysis, unlabeled PRL from 0.2 ng to 6.4 ng/tube was added in the reaction mixture. To determine the time-dependent PRL binding, the tubes were placed in an ice bath during the preparation and transferred to a water bath to start the reaction. The k_{+1} was calculated according to the methods described by Shiu and Friesen (1974). The k_{-1} was calculated according to the methods described by El-Refai (1984). Experiments were performed in triplicate, and each determination was repeated at least twice.

Affinity-labeling, SDS-PAGE and autoradiography

The procedures for affinity-labeling, SDS-PAGE and autoradiography have previously been described (Sakai and Ike, 1987). In brief, the microsomes (200 μ g) were incubated with 300,000 cpm of [125 I]PRL, as described above. The microsomes were washed once with the binding buffer and twice with 10 mM phosphate buffer (pH 7.4). The PRL-receptor complex was cross-linked with 0.75 mM disuccinimidyl suberate, and was denatured in the presence of 2% SDS

and 100 mM dithiothreitol. The complex was electrophoresed on a 10% polyacrylamide gel. The dried gel was used for autoradiography. The M_r of the PRL receptor was estimated with the SDS-PAGE M_r marker standards (Bio-Rad, Richmond, Calif.).

Results and Discussion

The effect of salts and their ionic strength on the binding of PRL was examined, and the results are shown in Fig. 1. The control values were obtained under metallic ion-free conditions. The amount of specific binding of [125 I]PRL was changed greatly by the type of salt and the ionic strength. All salts had biphasic effects on PRL binding proportional to their concentrations: there was a marked increase in

Fig. 1. Effect of various salts on PRL binding. The amount of specific binding of [125 I]PRL obtained in the absence of salt was about 2,530 cpm/tube, and this value was taken as 100%. The data represent the mean values obtained from two independent experiments. KCl (○—○), NaCl (●—●), $CaCl_2$ (○---○), $MgCl_2$ (●---●).

the PRL binding at lower concentrations and a decrease at higher concentrations. KCl and NaCl from 0 to 100 mM increased the binding about 1.6-fold. At higher concentrations, both monovalent salts decreased the amount of specific binding slightly. MgCl₂ and CaCl₂ concentrations at 0 mM to 25 mM enhanced the binding of PRL about 2.1- and 2.4-fold, respectively. At higher concentrations, the amount of specific binding decreased in a linear fashion and, at 500 mM, decreased to that of the 0 mM control. The results show that bivalent salts exert a greater effect on promoting the binding of PRL than monovalent salts. These results are consistent with findings in the rabbit mammary gland (Shiu and Friesen, 1974). The effects of salts on PRL binding were slightly different from rat liver PRL receptor (Silverstein and Richard, 1979; Webb *et al.*, 1986). This appears to be due to differences in the PRL-binding species: the M_r of PRL-binding components have been reported as 35,000–46,000 for rabbit liver (Haeuptle

et al., 1983; Hughes *et al.*, 1983) and rat liver (Borst and Sayare, 1982; Haldosen and Gustafsson, 1987; Katoh *et al.*, 1987). In the present experiments, the amount of specific binding obtained in the presence of 2 mM EDTA was comparable to that observed in the absence of salts. The amount of non-specific binding was always less than 1.2% of the total radioactivity added.

In the presence of various concentrations of the salts, the k_{+1} , k_{-1} , K_d , and binding capacity were determined from the data for the time-course kinetics and equilibrium binding. Table 1 shows the results obtained in the presence of NaCl, CaCl₂ and MgCl₂. Both mono- and bivalent salts had biphasic effects on the change in K_d . The addition of salts at low concentrations decreased the K_d for PRL binding and, higher concentrations caused an increase. The K_d was changed greatly in the presence of bivalent salts. A small K_d value was obtained in the presence of either NaCl at 100 mM or

Table 1. Effects of salts on changes in the k_{+1} , k_{-1} , k_d , and binding capacity. (mean $\pm$ SD, $n=3$)

Salt (mM)	k_{+1} (time-course kinetics) (nM ⁻¹ h ⁻¹)	k_{-1} (h ⁻¹ $\times 10^2$)	k_d (Scatchard analysis) (pM)	Capacity (fmol)
NaCl				
0	0.41 $\pm$ 0.05	6.46 $\pm$ 0.63	168 $\pm$ 14	10.2 $\pm$ 0.6
5	0.52 $\pm$ 0.04	6.63 $\pm$ 0.59	137 $\pm$ 12	11.0 $\pm$ 0.3
25	0.65 $\pm$ 0.11	7.02 $\pm$ 0.41	108 $\pm$ 15	11.2 $\pm$ 0.5
100	0.80 $\pm$ 0.09	6.89 $\pm$ 0.29	85 $\pm$ 11	10.9 $\pm$ 0.7
500	0.68 $\pm$ 0.07	6.91 $\pm$ 0.33	105 $\pm$ 12	11.2 $\pm$ 0.9
1000	0.51 $\pm$ 0.06	6.95 $\pm$ 0.53	111 $\pm$ 11	11.3 $\pm$ 1.1
MgCl ₂				
0	0.41 $\pm$ 0.03	6.74 $\pm$ 0.59	169 $\pm$ 13	10.1 $\pm$ 0.4
5	0.95 $\pm$ 0.13	6.85 $\pm$ 0.72	72 $\pm$ 15	11.3 $\pm$ 0.8
10	1.17 $\pm$ 0.09	7.07 $\pm$ 0.51	57 $\pm$ 9	14.4 $\pm$ 1.1
25	1.28 $\pm$ 0.11	6.83 $\pm$ 0.28	54 $\pm$ 10	16.2 $\pm$ 0.4
250	0.56 $\pm$ 0.02	6.11 $\pm$ 0.39	110 $\pm$ 11	16.1 $\pm$ 0.6
500	0.26 $\pm$ 0.04	5.82 $\pm$ 0.21	214 $\pm$ 8	15.8 $\pm$ 0.9
CaCl ₂				
0	0.42 $\pm$ 0.06	6.55 $\pm$ 0.32	158 $\pm$ 9	10.3 $\pm$ 0.4
25	1.44 $\pm$ 0.12	6.90 $\pm$ 0.49	48 $\pm$ 11	16.2 $\pm$ 0.4
250	0.90 $\pm$ 0.11	6.35 $\pm$ 0.21	74 $\pm$ 9	15.7 $\pm$ 0.8

bivalent salts at 25 mM. By increasing the NaCl concentration of from 0 M to 1 M, the binding capacity was unchanged and was comparable to the 0 mM control. The amount obtained in the presence of bivalent salts at 25 mM was about 1.6-fold larger than for the 0 mM control. The increased binding capacity was maintained with concentrations of MgCl_2 from 25 mM to 500 mM. The k_{+1} of PRL binding increased about 2.0-fold, when the NaCl concentration was increased from 0 mM to 100 mM. The k_{-1} was unaffected by NaCl concentrations between 0 M and 1 M. CaCl_2 and MgCl_2 at 25 mM enhanced the association rate about 3.4- and 3.1-fold, respectively. At higher than 250 mM, the k_{+1} decreased greatly. Ca^{2+} - or Mg^{2+} -dependent modulation of receptor affinity has been shown in other receptor systems (Anderson and Reichert, 1982; Antoni and Chadio, 1989; Loeb and Drickamer, 1988).

The apparent M_r of free PRL was about $26,000 \pm 1,200$ (mean $\pm$ SD, $n=4$) and has been subtracted from the M_r of the complex. As shown in Fig. 2, the mammary gland con-

tained three species of PRL-binding proteins. We confirmed our previous findings (Murakami *et al.*, 1988; Sakai and Ike, 1987). In the present study, the band of M_r 22,000 could also be detected in the lane of the noncross-linked control. The receptor with an M_r of $78,000 \pm 2,400$ or of $34,500 \pm 2,200$ receptor was specific for the binding of PRL. The M_r 34,500 receptor could be detected on the autoradiogram regardless of the presence of either mono- or bivalent salts. The M_r 78,000 receptor appeared on the autoradiogram in the presence of bivalent salts.

It is apparent that bivalent cations modulate the affinity as well as the availability of the receptor for PRL binding. The mechanism of the effect by bivalent cations is not well understood. As shown here, the introduction of bivalent cation accelerated the rate of association of PRL with the receptor greatly. The binding of PRL to the M_r 78,000 receptor occurred in the presence of Ca^{2+} or Mg^{2+} . Taken together, it appears that bivalent cation interacts with the PRL-binding site of the

Fig. 2. Autoradiography of covalently cross-linked PRL-receptor complex. Hundred μg of protein was applied to each lane. Radioactivity of total (and non-specific) binding was as follows; lane 1, control, 9,300 (560); lane 2, NaCl, 12,600 (480); lane 3, KCl, 13,300 (460); lane 4, MgCl_2 , 16,200 (1,100); lane 5, CaCl_2 , 17,000 (1,300); and lane 6, non-cross-linked samples of lane 5. The concentration of mono- or bivalent cation was at 100 mM or at 25 mM, respectively. The migrating positions of the high and low M_r protein standards are shown in the figure.

M_r 78,000 receptor to stabilize a conformation favoring the binding of PRL. At high concentrations, the k_{+1} decreased greatly in the presence of bivalent cations. This is probably due to the alteration of the reaction environment (Sakai and Suzuki, 1989). Both Ca^{2+} and Mg^{2+} ions at high concentrations are known to have a chaotropic action.

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