Rat Ovarian and Adrenal Prolactin Receptors. Sizes and Effects of Divalent Metal Ions.

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Abstract

Receptor fractions were prepared from follicle-rich ovaries (for FSH), luteal cell-rich ovaries (for LH and PRL), and adrenals (for PRL) of rats. Divalent metal ions, Mg++, Ca++, and Mn++ showed inhibitory effects on the bindings of LH and FSH to their receptors. The binding of the former was more sensitive to these ions than the latter. On the other hand they showed bell-shaped promotive effects on PRL-ovarian receptor binding, the maximal effects being observed at 10–20 mM. Besides these ions, Ba++ also had a promotive effect, while other divalent metal ions such as Zn++, Cd++, Ni++, and Co++ showed inhibitory effects on PRL-ovarian receptor binding at 5 mM. Mg++ and Ca++ also promoted PRL-adrenal receptor binding, while Mn++ promoted the binding at 10 mM but inhibited it at higher concentrations. Association constant (Ka) and binding capacity (Bmax) of PRL receptors of the ovary and the adrenal were significantly different (ovary: $Ka = 0.69 \times 10^{10}$ M$^{-1}$, $B_{\text{max}} = 62$ fmol/mg protein, adrenal: $Ka = 0.21 \times 10^{10}$ M$^{-1}$, $B_{\text{max}} = 99$ fmol/mg protein). Ka of the ovarian PRL receptor was not influenced by these divalent ions, while that of the adrenal receptor was doubled by Ca and Mn ions. Bmax of the latter was also increased. A cooperative effect of Mg and Ca ions was observed on Ka and Bmax of the adrenal receptor. The sizes of the PRL binding sites of these organs revealed by affinity labelling were 17 K and 40 K in the ovary, and 40 K and 110 K in the adrenal. These results indicate the different properties of receptors in these different target organs.

The first step in the action mechanism of a protein hormone is its binding to the target structure, the receptor. The hormone-receptor interaction has been found to be influenced by many factors such as ionic strength, pH, and temperature (Bhalla and Reichert, 1974). To clarify these influences is essential to the better understanding of the nature of the hormone-receptor interaction as well as the nature of the receptor itself. In particular, an anterior pituitary hormone, prolactin (PRL), has been shown to have multiple actions on multiple target organs, and it seems to be important to compare the nature of the receptors in the different target cells and their interaction with PRL. In the hormonal receptors which are associated with the adenylate cyclase system, some divalent cations are known to directly affect the hormone-receptor binding.

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(Bellorin-Font et al., 1982, Andersen and Reichert, 1982). PRL receptor, on the other hand, has been reported not to be related to the adenylate cyclase system (Turkington et al., 1973, Hunzicker-Dunn and Birnbaumer, 1976, Oka and Perry, 1976, Rillema, 1980). Little has been known about the relationship between divalent cations and the PRL-receptor binding.

In the present studies, we first compared the effects of divalent metal ions on the binding of PRL to the ovarian receptor with those on the binding of LH and FSH to their receptors in the same organ, and then compared the effects on the PRL binding to the receptors of different origins, the ovary and the adrenal gland, and also tried to estimate the molecular sizes of the PRL binding sites to find possible differences in the PRL receptors in these organs.

Materials and Methods

Hormones
Hormone preparations, NIADDK-oPRL-I-1 (AFP-4328), NIADDK-rat LH-I-5, and NIADDK-rat FSH-I-4, were supplied by the National Hormone and Pituitary Program, Baltimore, MD. Human chorionic gonadotropin (hCG, Pregnyl) was purchased from Sankyo Pharm. Co., Ltd., Tokyo, and pregnant mare’s serum gonadotropin (PMSG, Serotropin) was obtained from Teikoku Hormone Mfg. Co., Ltd., Tokyo.

Animals
Female rats of Holtzman strain were obtained at 24-26 days of age from the Institute of Experimental Animal Research, Gunma University School of Medicine. They received single subcutaneous injections of 50IU PMSG, and, 3 days later, some of them were sacrificed to collect follicle-rich ovaries for FSH receptor preparation (Wakabayashi et al., 1980). Other PMSG-treated rats were further injected with 25IU hCG 3 days later, and the luteal cell-rich ovaries were harvested another 7 days later to obtain LH and PRL receptor preparations (Lee and Ryan, 1973). Intact female rats of the same strain were also employed at the age of 42-45 days as donors of the adrenal PRL receptor preparation.

Preparation of crude receptor fractions
The membrane receptor fractions were prepared according to the method of Gospodarowicz (1973) with minor modification. The tissue was weighed and minced finely, and homogenized in 10 volumes of ice-cold 5 mM Tris-HCl buffer, pH 7.4, containing 0.5 M MgCl₂ and 0.25M sucrose with 20 strokes of a Dounce type homogenizer. The homogenate was centrifuged at 500×g for 5 min under refrigeration.

The supernatant fluid was collected, and the pellet was resuspended in the same volume of the buffer, and further homogenized by 5 strokes, then centrifuged. After this treatment was repeated twice, the supernatants and the pellet were combined, and added to 3 times the amount of the buffer, then centrifuged at 6,000×g for 30 min. The pellet was washed with 5mM Tris-HCl-0.5 mM MgCl₂, pH 7.4, and resuspended in the same buffer. The suspension was added to 70% sucrose-Tris-MgCl₂ buffer to make the final concentration of sucrose 48%. Five ml of this suspension was placed in a cellulose nitrate tube, and was overlaid with 10 ml of 45% sucrose-Tris-MgCl₂, 10 ml of 41% sucrose-Tris-MgCl₂, and 5 ml of 37% sucrose-Tris-MgCl₂, then centrifuged at 100,000×g for 90 min. The membrane fragments which were between 41% and 37% sucrose was collected, washed twice with 10 volumes of Tris-HCl buffer and centrifuged at 6,000×g for 15 min, then resuspended in the same buffer and stored at −80°C until use. The protein content was determined by the method of Lowry et al. (1951).

Receptor binding studies
Incubation was carried out in a 12×75 mm polypropylene tube containing 250 µl of the reaction mixture, pH 7.4, consisting of 0.5% bovine serum albumin (BSA), 50 mM Tris-HCl, 300 µg of receptor protein fraction, varying concentrations of metal ions, and ¹²⁵I-labelled hormones prepared by the lactoperoxidase method (Miyachi et al., 1972). After overnight incubation at 20°C, 2 ml of 0.5% BSA-Tris-HCl was added to the reaction mixture, then the tubes were centrifuged at 20,000×g for 15 min, and the precipitates were counted for radioactivity. Non-specific bindings were estimated in the presence of 1 µg oPRL, 50IU hCG, or 50IU PMSG for PRL, LH, or FSH receptor binding, respectively.
PRL-receptor cross-linking

PRL-receptor cross-linking was carried out according to the method of Rebois et al. (1981), with minor modification. PRL receptor fraction, equivalent to 500 µg membrane protein, was incubated with 125I-labelled oPRL for 18 hrs at 4°C in 250 µl of PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.5), and centrifuged at 20,000 × g for 15 min. The precipitates were resuspended in 500 µl of PBS, and added to the same volume of 1 mM disuccinimidyl suberate (DSS, Pierce Chem. Co.) dissolved in dimethyl sulfoxide. After incubation at 0°C for 15 min, the reaction was terminated by adding 2 ml of Tris-HCl buffer, then centrifuged at 15,000 × g for 15 min. The cross-linked hormone-receptor complex obtained as a pellet was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and autoradiography

SDS-PAGE was carried out with a vertical slab gel unit (Marisol Co., Tokyo) by the method of Laemmli (1970). The samples were solubilized in 100 µl of 2% SDS containing 10% glycerol, and 0.001% bromophenol blue by heating at 100°C for 1 min, and the insoluble residue was removed by centrifugation. For reduction of disulfide bonds, the solution was added with dithiothreitol (DTT) at a final concentration of 50 mM. The samples were applied on 10% polyacrylamide gel 2 mm thick, and electrophoresed under a constant current of 24 mA/gel. The gel was dried and an autoradiogram was prepared with Kodak XAR-5 film (Eastman Kodak, N.Y.), then scanned with a densitometer (Shimadzu CS-930 TLC scanner). For calibration of the molecular size, SDS-PAGE Standards (BIO-RAD Laboratories) were employed.

![Graph showing effects of Mg, Ca, and Mn ions on the bindings of PRL, LH, and FSH to their ovarian receptors.](image-url)
Statistical analyses

Effects of treatments were analyzed by one-way analysis of variances, and comparison of the two groups was carried out by either Student's t-test or Cochran-Cox test depending on the variance ratio. Multiple comparisons were made by Duncan's new multiple range test after Bartlett's test for the uniformity of variances.

Results

Effects of divalent metal ions on the binding of PRL, LH, and FSH to their ovarian receptors

As shown in Fig. 1, divalent metal ions such as Mg++, Ca++, and Mn++ showed different effects on the hormone binding to the receptors though they are derived from the same organ, the ovary.

Binding of PRL to the receptor was promoted by these ions with the optimal concentrations of 12.5 mM, 50 mM, and 25 mM for Mg++, Ca++, and Mn++, respectively.

On the other hand, effects of these ions were inhibitory on LH and FSH binding. They inhibited LH-receptor binding with similar potencies, and the inhibition was observed at far lower concentrations (50% inhibition at 6 mM) than those on FSH-receptor binding where 50% inhibitory concentrations were 62, 44, and 23 mM for Mg++, Ca++, and Mn++, respectively.

Effects of divalent metal ions on the binding of PRL to the ovarian receptor

Fig. 2 shows the effects of a wider variety of divalent metal ions on the binding. All the ions were used in the form of chloride at a concentration of 5 mM. Besides Mg++, Ca++, and Mn++, only Ba++ showed a promoting effect, while Zn++, Cd++, Ni++, and Co++ inhibited the binding.

Scatchard plot analyses (Scatchard, 1949)
carried out in the presence (10 mM) and absence of Mg$^{++}$, Ca$^{++}$, and Mn$^{++}$, indicated that no significant changes in the association constant (Ka) were caused by these metal ions and that the binding capacity (Bmax) was significantly decreased by Mn$^{++}$ (Table 1).

Results of affinity labelling employing $^{125}$I-PRL in the presence (10 mM) and absence of Mg$^{++}$ followed by DSS treatment, SDS-PAGE, and autoradiography are shown in Fig. 3. Specific radioactive spots which reflect the binding sites of PRL were found at the positions of 62K and 39K daltons, showing the presence of two kinds of PRL binding sites with molecular sizes of 40K and 17K. In the presence of Mg ion, the ratio of PRL found at 62K and 39K was 79:21, while in the presence of 10 mM of Mg$^{++}$ the ratio became 65:35, indicating an increased ratio of PRL which bound to the smaller size binding site of 17K.

Table 1. Effects of metal ions on the binding of PRL to the ovarian receptor.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Ka ($10^{10}$M$^{-1}$)</th>
<th>Bmax (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metal Free</td>
<td>0.69±0.03</td>
<td>62±1.5</td>
</tr>
<tr>
<td>CaCl$_2$ (10 mM)</td>
<td>0.77±0.11</td>
<td>57±4.8 **</td>
</tr>
<tr>
<td>MnCl$_2$ (10 mM)</td>
<td>0.78±0.10</td>
<td>42±3.4</td>
</tr>
<tr>
<td>MgCl$_2$ (10 mM)</td>
<td>0.74±0.12</td>
<td>62±5.1</td>
</tr>
</tbody>
</table>

Association constant and Bmax were calculated from 5 points and shown with S.E.
No significant effect on Ka by one-way analysis of variances.
**: p<0.01

Fig. 3. Autoradiogram showing the PRL binding sites in the ovarian membrane preparation. Labelled PRL was incubated with the membrane preparation in the presence (lane a, c) and absence (lane b, d) of 10 μg of unabelled PRL with 10 mM MgCl$_2$ (lane a, b) and without the ion (lane c, d), followed by cross-linking with DSS and by SDS-PAGE. Densitometric scanning patterns of the lanes are shown in the left side.
Fig. 4. Scatchard plot analyses of PRL bindings to ovarian and adrenal membrane preparations. $K_a_1$, MaxB$_1$, (●): ovary. $K_a_2$, MaxB$_2$, (○): adrenal gland.

Fig. 5. Effects of Mg, Ca and Mn ions on the PRL binding to the adrenal receptor. □: MgCl$_2$, ●: CaCl$_2$, ○: MnCl$_2$
Effects of divalent metal ions on the binding of PRL to the adrenal receptor

Another PRL receptor fraction was obtained from the rat adrenals, and its association constant and binding capacity were estimated by Scatchard plot analysis. As shown in Fig. 4, the adrenal PRL receptor had a lower association constant (p<0.001) and a higher binding capacity (p<0.05) than those of the ovarian PRL receptor.

Fig. 5 shows the effect of varied concentrations of Mg++, Ca++, and Mn++ on the PRL binding to the adrenal preparation. Quite different from the case of the ovarian receptor, Mg++ and Ca++ exhibited similar promotive effects on the binding, and the effect increased as the concentration increased up to 100 mM, and thereafter slightly decreased, while Mn++ caused promotion around 10 mM and inhibition at higher concentrations.

Table 2. Effects of metal ions on the binding of PRL to the adrenal receptor.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Ka (10^10 M^-1)</th>
<th>Bmax (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metal Free</td>
<td>0.21±0.024*</td>
<td>99±7.2</td>
</tr>
<tr>
<td>CaCl₂ (10 mM)</td>
<td>0.40±0.064</td>
<td>116±13.1</td>
</tr>
<tr>
<td>MnCl₂ (10 mM)</td>
<td>0.43±0.015***</td>
<td>149±3.40**</td>
</tr>
<tr>
<td>MgCl₂ (10 mM)</td>
<td>0.28±0.045</td>
<td>a 108±11.5</td>
</tr>
<tr>
<td>MgCl₂ (10 mM)</td>
<td></td>
<td>1.01±0.107**</td>
</tr>
<tr>
<td>CaCl₂ (10 mM)</td>
<td></td>
<td>a 198±11.2**</td>
</tr>
</tbody>
</table>

Association constant and Bmax were calculated from 5 points and shown with S.E.

*: p<0.05, **: p<0.01, ***: p<0.001 vs metal free.

Fig. 6. Autoradiogram showing PRL binding sites in the adrenal membrane preparation. Labelled PRL was incubated in the presence (lane a, b, c) and absence (lane A, B, C) of 10 μg of unlabelled PRL, and with 10 mM of ions (A, a: MgCl₂, B, b: MnCl₂, C, c: CaCl₂), followed by DSS treatment and SDS-PAGE.
adrenal PRL receptor, Ca\textsuperscript{++} and Mn\textsuperscript{++} caused a significant increase in the association constant, and Mn\textsuperscript{++} also caused a significant increase in the binding capacity. Mg\textsuperscript{++} alone showed only slight effects on $K_a$ and $B_{\text{max}}$. However, when it was added to Ca\textsuperscript{++}, significant increases were observed over those with Ca\textsuperscript{++} alone, suggesting some cooperative effects of these ions on the PRL-receptor binding.

**Molecular size of the adrenal PRL binding sites**

Fig. 6 shows the results of affinity labelling of the PRL-binding sites. The specific binding spots of radioactive PRL were found at 62K and 135K, but not at 39K which was one of the PRL-binding sites in the ovary. These facts indicated that the adrenal gland had a binding site of large molecular size of approximately 100K besides the 40K site which was of the same size as the ovarian binding site but did not have the 17K binding site.

**Discussion**

PRL exerts numerous biological actions which are classified by Nicoll (1981) into the following seven categories: 1) actions related to water and electrolyte balance, 2) growth-promoting and developmental effects, 3) metabolic effects, 4) actions related to reproduction, 5) effects on integumentary and ectodermal structures, 6) synergistic and antagonistic actions with steroid hormones, and 7) synergistic and antagonistic action with thyroid hormones. This means that PRL has a variety of target organs and so binds to many kinds of cells. Then, there is the question whether the PRL receptors in different target cells have similar properties or have a different structure and interact differently with PRL corresponding to the multiple hormonal actions. Elucidation of this problem will make it possible to understand the hormone-receptor relationship better.

In our present studies, we obtained evidence indicating that PRL receptors in the different target organs may have a different structure, by comparing the affinity constant, binding capacity, the effects of divalent metal ions on the PRL-receptor binding, and the molecular size.

Our first finding was that the effects of divalent metal ions on the binding of PRL, LH, and FSH to their receptors obtained from the same organ, the ovary, were quite different. This may indicate the dependence of the effects of these ions on the structure of hormone and/or receptor. In particular, the binding of LH to its ovarian receptor was more sensitive than that of FSH, being inhibited at far lower concentrations of these ions than those for the inhibition of FSH-receptor binding. Andersen and Reichert (1982) reported the promotive effects of Mg, Ca, and Mn ions on FSH-rat testicular receptor binding at 2–5 mM. Our observation with the ovarian receptor, on the other hand, revealed only the inhibitory effects of these ions at concentrations over 10 mM. Sanzo and Reichert (1982) showed a positive effect of Ca\textsuperscript{++} on the binding of LH and the testicular receptor at low concentrations, while Lee and Ryan (1972) reported that higher concentrations of Mg\textsuperscript{++} and Ca\textsuperscript{++} over 15 mM inhibited the binding of LH and its ovarian receptor, but that their low concentrations did not affect the binding. These facts seem to indicate that the effects of the divalent ions on the binding a hormone to its receptor are different if the receptors are derived from different organs.

Compared with LH and FSH, the binding of PRL to the ovarian receptor was influenced by these divalent metal ions in a bell-shaped mode. The potencies of these three ions were not equal. The optimal concentrations of Mg\textsuperscript{++}, Ca\textsuperscript{++}, and Mn\textsuperscript{++} were 12.5, 50, and 25 mM, respectively. Ca\textsuperscript{++} showed the strongest effect at its optimal
concentration followed by Mg++, then Mn++. Not all of the divalent metal ions promotes PRL-receptor binding, and besides Mg++, Ca++, and Mn++, only Ba++ showed a promotive effect.

Besides the ovary, the mammary gland and the liver are well known target organs of PRL. But these organs are not always suitable for receptor analysis because of the abundance of fat, difficulty of isolation of the gland, and influence of proteolytic enzymes, etc. In our present studies, therefore we used the ovary and the adrenal gland, because they are easy to treat. Waters et al., (1984) compared the rabbit PRL receptors from different organs, and reported that the ovarian PRL receptor had the highest association constant.

On the other hand, PRL has been known to modulate the secretion of adrenal cortical steroids, namely, it inhibits adrenal 5α-reductase which catabolizes corticosterone and increases the responsiveness to the stimulation by ACTH (Witorsch and Edwards, 1975). It has also been observed that the adrenals of rats and rabbits have high levels of binding sites (Franz et al., 1974, Posner et al., 1974, Marshall et al., 1975, 1978, 1979, and Oregebin-Crist and Djiane, 1979). As shown in Fig. 4, the adrenal PRL receptor is different from the ovarian receptor in both association constant (Ka) and binding capacity (Bmax), i.e. it has a lower affinity and a higher capacity. The association constants calculated by Scatchard plot analyses were of the same order as those reported by investigators concerning the mammary gland and the liver (Shiu and Friesen, 1974, Liscia et al., 1982b, Borst and Sayares, 1982).

The effects of divalent metal ions on these receptors with different affinity and binding capacity were, as shown in Fig. 5, not equal. That is, on the adrenal receptor, the maximal effects of Mg and Ca ions were observed at higher concentrations than on the ovarian receptor. Also, at 10 mM, the order of the potencies of these metal ions was Ca++ > Mg++ > Mn++ on the ovarian receptor, while on the adrenal receptor, the order was Mn++ > Ca++ > Mg++, and at concentrations higher than 50 mM, the inhibitory effect of Mn++ was manifested.

Whether or not these divalent metal ions which have the promotive effect on PRL-receptor binding have any influence on the association constant or binding capacity is an interesting problem. If we compare the results obtained by Scatchard plot analyses, shown in table 2, we can also observe their different effects on these receptors of different origins. The association constant of the ovarian receptor, which is somewhat higher than the adrenal receptor, was not influenced by the divalent ions, and the binding capacity was decreased only by Mn++. The increased binding of the hormone caused by the divalent ions without changing the association constant might occur through the change in the localization of the receptors on the cell membrane to make them scatter more widely, minimizing the geographical interference and causing an increase in practically effective binding sites to the hormone. It must be also taken into consideration that the present studies were carried out employing the cell-free membrane preparations. On the other hand, the association constant of the adrenal receptor was increased to about twice by Ca and Mn ions, and the binding capacity was also increased by Mn++ by about 50%. Neither Ca++ nor Mg++ significantly affected Bmax, and Mg++ did not significantly increase Ka. However, in the presence of both ions, Ka and Bmax were markedly increased. These facts seem to be further evidence of the difference of the nature of PRL receptors in these organs. Especially, if we consider the usual Ca++ and Mg++ concentrations in the blood to be around 2.5 mM and 1 mM, respectively, the cooperative effect of Mg and Ca ions may be related to the physiological regulation possibly by changing the configuration and the distribution of the
PRL receptors on the cell membrane to increased Ka and Bmax.

The difference in the molecular sizes of the binding sites is also noteworthy. In the ovary, we could observe 17K and 40K dalton binding sites. The presence of the smaller receptor of 17K was reported by Haeuptle et al. (1983) also in the rabbit mammary gland. A receptor of around 40K size has been observed by many investigators in the liver and the mammary gland as the major PRL binding site (Haeuptle et al., 1983, Hughes et al. 1983, Liscia et al., 1982a, 1982b, Borst and Sayare, 1980). In the adrenal gland, the small 17K binding site was not observed, but besides the 40K site which was the same size as the one in the ovary, a larger binding site of 110K was found. The presence of this larger size binding site has been reported by Bonifacino and Dufau (1983) in the liver, and also by Hughes et al. (1983) in [M-9] lymphoma cells. Our observation about the size of the binding sites indicated that, in these organs, the structure of the PRL receptors or at least the population of subgroups of the receptor may be different, though more detailed studies are needed to clarify the nature of these binding sites.

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References


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