Prostaglandin F$_{2\alpha}$ receptor in the neurohypophysis of hens

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ABSTRACT

To elucidate whether the receptor for prostaglandin (PG) F$_{2\alpha}$, one of PG, exists in the neurohypophysis in hens and whether the binding of receptor changes with relation to oviposition, the PGF$_{2\alpha}$ binding component in the membrane fraction of the neurohypophysis of laying hens was analyzed by radioligand binding assay using [5,6,8,9,11,12,14,15(n)-$^3$H]PGF$_{2\alpha}$. The binding component had characteristics of a receptor such as binding specificity, high affinity, and limited capacity for PGF$_{2\alpha}$. Scatchard analysis indicated that the binding site was of a single class. The binding capacity of the receptor was smaller in laying hens than in nonlaying hens, whereas the binding affinity was not significantly different between these hens. When nonlaying hens received an i.m. injection of estradiol-17β or progesterone (0.5 mg/hen), the specific binding of the PGF$_{2\alpha}$ receptor in the neurohypophysis was decreased. In laying hens, the specific binding decreased and the blood arginine vasotocin (AVT) concentration increased just after oviposition but did not change during a 24-h day in nonlaying hens. An i.v. injection of PGF$_{2\alpha}$ (2 µg/hen) induced oviposition and caused an increase in the blood AVT concentration with a decrease in the specific binding of PGF$_{2\alpha}$ receptor. The present study suggests a possibility that PGF$_{2\alpha}$ may directly cause the AVT release from the neurohypophysis at oviposition time in hens.

Key words: hen neurohypophysis, prostaglandin F$_{2\alpha}$ receptor, receptor binding, arginine vasotocin release, oviposition

INTRODUCTION

Prostaglandins (PG) are produced in various tissues and have been thought to be a local hormone (Bentley, 1976). Prostaglandin F$_{2\alpha}$ belongs to the PGF series, which is one of PG consisting of 10 forms (PGA through PGJ; Maclouf et al., 1977; Fukushima et al., 1982; Wolfe, 1982; Fukushima, 1990). An injection of PGF$_{2\alpha}$ into laying hens induces oviposition with an increase in the blood concentration of arginine vasotocin (AVT; Shimada et al., 1986, 1987; Murakami et al., 1990), which is one of neurohypophysial hormones in the avian (Munsick et al., 1960). The blood concentration of AVT is higher in laying hens than in nonlaying hens (Takahashi et al., 1994b), but whether it relates to the PGF$_{2\alpha}$ is obscure. The AVT possesses the effect to induce oviposition in hens (Rzasa and Ewy, 1970), and at the time of spontaneous oviposition, the AVT concentration in blood increases (Sturkie and Lin, 1966; Tanaka et al., 1984; Rice et al., 1985; Shimada et al., 1986; Takahashi et al., 1994a). The specific binding component for PGF$_{2\alpha}$ exists in the uterus of hens (Toth et al., 1979), and PGF$_{2\alpha}$ possesses an effect of causing contractions of the uterine smooth muscle (Wechsung and Houvenaghel, 1976; Olson et al., 1978; Shimada and Asai, 1979). Physical stimulation by the palpation increases the AVT concentration in blood (Shimada et al., 1987). Because the PGF content in the uterine tissue of laying hens increases at the time of spontaneous oviposition (Takahashi et al., 2004), it is thought that the PGF may act as a local hormone to the uterus at this time. The blood concentration of PGF also increases at the time of spontaneous oviposition (Saito et al., 1987; Shimada et al., 1987; Takahashi et al., 1999) with an increase in the content of the ovarian preovulatory follicle (Saito et al., 1987; Shimada et al., 1987). It is thought from these facts that contractions of the uterine musculature are caused by the direct action of PGF$_{2\alpha}$ on the uterus, and a message is transmitted from the uterus to the central nervous system via a neural pathway, and an abrupt release of AVT from the neurohypophysis is caused. However, a possibility of the direct action of PGF$_{2\alpha}$ on the neurohypophysis has not been examined. In rats, it was reported that the PGF$_{2\alpha}$ increases vasopressin and oxytocin releases from isolated neurointermediate lobes in vitro (Bojanowska and Guzek, 1989). The present study was performed to obtain evidence for a direct action of PGF$_{2\alpha}$ on the neurohypophysis of the hen as indicated by the presence of a receptor and, if obtained, to determine whether the
bindings of PGF₂α receptor are different between laying hens and nonlaying hens and to determine whether PGF₂α binding to the receptor changes with relation to oviposition.

**MATERIALS AND METHODS**

**Birds and Tissues**

White Leghorn laying and nonlaying hens (20 mo of age; 1.8 to 2.2 kg of BW) were obtained from a flock of approximately 1,200 hens kept in individual cages under 14 h (0500 to 1900 h) light per day with feed (15% CP; 2,800 kcal of ME; Japan Feeding Standard for Poultry, 1992) and water provided for ad libitum consumption. All birds were cared for and used according to the institutional guidelines of Gifu University. The laying hens used were those laying 4 to 6 sequential eggs with a 1-d pause between sequences for more than 2 wk, and nonlaying (moltig) hens used were those that had not laid an egg at least for 10 d before experiments. The weights of ovary and oviduct of the nonlaying hens were less than 8.8 and 6.3 g, respectively. The serum concentrations of the ovarian steroid hormones were less than 306 pM (estradiol-17β; E₂), 325 pM (progesterone; P₄), and 319 pM (testosterone), respectively, as measured by routine RIA (Shodono et al., 1975).

In the first experiment, laying hens were killed by decapitation at 1000 h regardless of the time of oviposition, and nonlaying hens were killed at the same hour of the day. The neurohypophysis (neural lobe of the hypothalamus; 1.9 to 2.2 mg per hen) was excised. Fifty tissues of the neurohypophysis were pooled and used as 1 sample for the binding assay to [³H]PGF₂α. ([³H]PGF₂α; Amersham International plc, Buckinghamshire, UK).

In the second experiment, nonlaying hens received an i.m. injection of E₂ (Sigma Chemical Co., St. Louis, MO), P₄ (Sigma Chemical Co.), 5α-dihydrotestosterone (DHT; E. Merck, Darmstadt, Germany), or vehicle (olive oil) at 1000 h. The dose of the steroids was 0.5 mg/0.5 mL per hen. The hens injected were killed 1 or 2 h after the injection. Fifteen tissues of neurohypophysis were pooled and used as a sample for the binding assay to [³H]PGF₂α.

In the third experiment, the time before oviposition was estimated from the oviposition time observed before the experiment. The clock time of oviposition of the first egg of the laying sequence was 0657 h ± 5 min (mean ± SEM, n = 50). Laying hens were killed at 6 different times (16, 14, 11, 6, and 3 h and 15 min) before expected oviposition of the first egg of the laying sequence and 3 other different times (within 1 min, 2 and 5 h) after oviposition. Nonlaying hens were also killed at 6 different times (1500, 2000, 0100, 0400, 0700, and 1200 h) corresponding to the time of sampling in laying hens during a 24-h day. Fifteen tissues of neurohypophysis were pooled as 1 sample at each time for the binding assay to [³H]PGF₂α.

In the fourth experiment, laying hens received an i.v. injection of PGF₂α (2 µg/hen) or saline vehicle (0.5 mL/hen) at 3 h before expected oviposition of the first egg of the laying sequence. The hens were killed at 1 min, approximately 3 min (within 1 min after induced oviposition), 5 min, and 10 min after the injection. Fifteen tissues of neurohypophysis were pooled as 1 sample for the binding assay to [³H]PGF₂α. Blood was collected from the wing vein in 6 hens, and serum concentrations of AVT were measured by a routine RIA (Goto et al., 1986; Takahashi et al., 1994a,b).

**Preparation of Membrane Fraction**

Tissues of the neurohypophysis were washed in ice-cold saline, blotted with a filter paper, weighed, and used immediately for preparation of the membrane fraction. The membrane fraction was prepared using the method of Kawashima et al. (1995) with slight modifications. All steps were performed at 4°C. Tissues of the neurohypophysis were homogenized in 15 vol/wt Tris-CaCl₂ buffer (TC; 50 mM Tris (Kishida Chemical Co. Ltd., Osaka, Japan)-HCl, 2 mM CaCl₂ (Wako Pure Chemical Industries Ltd., Osaka, Japan), pH 7.4) by using a Potter-Elvehjem-type glass Teflon homogenizer (Takashima System Ltd., Tokyo, Japan) with 10 strokes while cooling in an ice-water bath. The homogenate was centrifuged at 700 × g for 10 min, and the supernatant was obtained. The precipitate was rehomogenized in the same buffer and recentrifuged. The pooled supernatants were centrifuged at 30,000 × g for 30 min. The precipitate was washed with the same centrifugation, and the precipitate was suspended in the TC buffer and used as a membrane fraction. The fraction was stored at −80°C until assayed. The protein concentration of the membrane fraction was measured by the method of Lowry et al. (1951) using BSA (Fraction V, Seikagaku Corp., Tokyo, Japan) as a standard.

**Binding Assay**

The specific binding of [³H]PGF₂α was measured by using the method of Takahashi et al. (1992, 1998) with slight modifications. Aliquots of the membrane fraction (15 µg of protein/tube) were incubated at 4°C for 60 min with [³H]PGF₂α (1 to 14 nM) in the absence (for total binding) or presence (for nonspecific binding) of 35 µM of unlabeled PGF₂α in a total volume of 300 µL. In the binding assay, 1.5-mL polypropylene microtubes (Treff AG, Degersheim, Switzerland) pretreated with TC buffer containing 1% BSA for 1 night at 4°C were used. For binding specificity, membrane fraction was incubated with [³H]PGF₂α (7 nM) in the absence or presence of various molar excess (7, 70, 700, or 7,000 nM) of unlabeled PG. The unlabeled PG used were PGF₂α, PGA₁, PGE₁, PGE₂, and 6-keto-PGF₁α. All of the unlabeled PG were purchased from Ono Pharmaceutical Co. Ltd. (Osaka, Japan). After the incubation, the tubes were centrifuged (10,000 × g, 20 min, 4°C) and the tubes were centrifuged (10,000 × g, 20 min, 4°C).
Specific $[^3H]$PGF$_2\alpha$ binding (fmol/mg of protein)  

<table>
<thead>
<tr>
<th>Addition</th>
<th>Specific $[^3H]$PGF$_2\alpha$ binding (fmol/mg of protein)</th>
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<tbody>
<tr>
<td>None</td>
<td>84.7 ± 2.2$^{A,2}$</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>167.0 ± 5.3$^{\mu}$</td>
</tr>
<tr>
<td>EDTA</td>
<td>87.1 ± 1.0$^{A}$</td>
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</tbody>
</table>

$^{A,2}$Means with different superscripts differ significantly ($P < 0.01$) by Newman-Keuls’ test.

$^1$Samples (15 µg/tube) were incubated in 50 mM Tris buffer (pH 7.4) containing 2 mM CaCl$_2$ or 2 mM EDTA at 30°C for 60 min with 7 nM [5,6,8,9,11,12,14,15(n)-$[^3H]$]PGF$_2\alpha$ ($[^3H]$PGF$_2\alpha$) in the absence or presence of 35 µM unlabeled PGF$_2\alpha$, and the specific $[^3H]$PGF$_2\alpha$ binding was measured.

$^2$Mean ± SEM of 3 separate pools of samples.

and the precipitate was rinsed with 500 µL of ice-cold TC buffer and recentrifuged. Liquid scintillation cocktail (ACS II, Amersham International Plc) was added to the precipitate, and the radioactivity was measured by using a liquid scintillation analyzer (Tri-Carb Model 1600TR, Packard Instrument Co., Meriden, CT). The counting efficiency of the analyzer for $^3$H was 46 to 54%. Specific binding was obtained by subtracting non-specific binding from total binding and expressed as moles per milligram of protein. The equilibrium dissociation constant ($K_d$) and maximum binding capacity ($B_{max}$) were determined by the method of Scatchard (1949).

### Preliminary Experiments

Relationships of specific $[^3H]$PGF$_2\alpha$ binding to the presence of a cation (2 mM of Ca$^{2+}$) and a chelator (2 mM EDTA), incubation time (10 to 480 min) and temperature (4 and 30°C), and protein concentration (6.25 to 50 µg per tube) were examined. The specific $[^3H]$PGF$_2\alpha$ binding was increased by the presence of 2 mM Ca$^{2+}$ but was not changed by the presence of EDTA.

### Binding Specificity

The $[^3H]$PGF$_2\alpha$ binding in the membrane fraction of the neurohypophysis in laying hens was markedly reduced by the presence of a 100-fold molar excess (700 nM) of unlabeled PGF$_2\alpha$ but was not affected by the presence of an equivalent molar concentration of unlabeled PGA$_1$ or PGE$_1$ (Figure 2). The unlabeled 6-keto-PGF$_1\alpha$ and PGE$_2$ reduced the binding (9 and 48%, respectively) when a 1,000-fold molar excess (7,000 nM) was used.

### Binding Affinity and Capacity

Specific $[^3H]$PGF$_2\alpha$ binding in the membrane fraction of the neurohypophysis of laying hens increased (Table 1). The specific binding at 30°C increased during the first 40 min of incubation and then reached a plateau up to 480 min. The specific binding at 4°C incubation increased during the first 240 min of incubation and reached a plateau up to 480 min, but the specific binding was lower than that at 30°C incubation (Figure 1). A linear increase in the specific binding with the increase in the protein concentration from 6.25 to 50 µg per tube was observed when incubated at 30°C for 60 min (data not shown). Based on these findings, the following experimental conditions were used in the present experiments: presence of 2 mM CaCl$_2$, 30°C incubation for 60 min, and 15 µg of protein per tube.

### Statistical Analyses

Student’s t-test was used to assess the significance of difference between 2 means. For comparisons among more than 2 groups, the data were analyzed by 1-way ANOVA (Snedecor and Cochran, 1967). When significant ($P < 0.05$) effects were found, Newman-Keuls’ multiple range test (Snedecor and Cochran, 1967) was used to separate means.

### RESULTS

#### Binding Specificity

Table 2. Equilibrium dissociation constant ($K_d$) and maximum binding capacity ($B_{max}$) of the prostaglandin F$_2\alpha$ (PGF$_2\alpha$) receptor in the plasma membrane fraction of the neurohypophysis of laying and nonlaying hens$^1$

<table>
<thead>
<tr>
<th>Hen</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}$ (fmol/mg of protein)</th>
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<tbody>
<tr>
<td>Laying</td>
<td>3.02 ± 0.08$^1$</td>
<td>154 ± 5$^{***}$</td>
</tr>
<tr>
<td>Nonlaying</td>
<td>3.08 ± 0.11</td>
<td>420 ± 12</td>
</tr>
</tbody>
</table>

$^1$Laying hens were killed at 1000 h without regard to the time of oviposition, and nonlaying hens were killed at the same hour of the day. Samples (15 µg per tube) were incubated at 30°C for 60 min with [5,6,8,9,11,12,14,15(n)-$[^3H]$]PGF$_2\alpha$ ($[^3H]$PGF$_2\alpha$, 1 to 14 nM) in the absence or presence of unlabeled PGF$_2\alpha$ (35 µM).

$^2$Calculated by Scatchard analysis.

$^3$Mean ± SEM of 5 separate pools of samples.

$^{**}$Significantly different ($P < 0.001$) from the value of nonlaying hens by t-test.

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Table 1. The specific $[^3H]$prostaglandin F$_2\alpha$ (PGF$_2\alpha$) binding$^1$ in the plasma membrane fraction of the neurohypophysis of laying hens in the absence or the presence of 2 mM of Ca$^{2+}$ or EDTA

<table>
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when increasing amounts of [3H]PGF2α were added (i.e., when amounts of free [3H]PGF2α were increased), and saturable at about 7 nM (Figure 3). Scatchard analysis revealed a linear relationship between the amount of [3H]PGF2α binding and the ratio of specific [3H]PGF2α binding to free [3H]PGF2α (Figure 3), indicating a single class of binding sites.

Table 2 shows the average of Kd and Bmax values obtained by Scatchard analysis in the membrane fraction of laying and nonlaying hens. The Kd value was not significantly different between the laying and nonlaying hens. The Bmax value was significantly smaller in laying hens than in nonlaying hens.

Changes in PGF2α Binding After E2, P4, and DHT Injection

The specific [3H]PGF2α binding in nonlaying hens showed a decrease at 1 h after an injection of E2 and P4 (Figure 4). An injection of DHT did not cause the change in the specific [3H]PGF2α binding (Figure 4).
Changes in PGF$_{2\alpha}$ Binding and Serum AVT Concentration Before and After Oviposition

The specific $[^3H]$PGF$_{2\alpha}$ binding and the serum AVT concentration in laying hens did not change from 16 h before oviposition to just before oviposition (15 min before expected oviposition), but the specific binding decreased and the AVT concentration increased just after oviposition (Figure 5). In nonlaying hens, neither the specific binding nor the AVT concentration changed during a 24-h day.

Changes in PGF$_{2\alpha}$ Binding and Serum AVT Concentration After PGF$_{2\alpha}$ Injection

The specific $[^3H]$PGF$_{2\alpha}$ binding in laying hens showed a decrease 1 min after an i.v. injection of PGF$_{2\alpha}$. The

serum concentration of AVT showed an increase approximately 3 min after the injection (within 1 min after the induced oviposition; Figure 6).

DISCUSSION

The membrane fraction of the neurohypophysis of the laying hen was found to contain a specific PGF$_{2\alpha}$-binding component (Table 1 and Figure 1). The binding component showed binding specificity to PGF$_{2\alpha}$ (Figure 2) and saturable binding (Figure 3). The $K_d$ value (moles per litter) obtained by Scatchard analysis was of the order of $10^{-3}$, and the $B_{\text{max}}$ value (moles per milligram of protein) was of the order of $10^{-13}$. The binding specificity, the high affinity (small $K_d$ value), and the limited capacity ($B_{\text{max}}$) serve as the requisites

\begin{figure}
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\includegraphics[width=\textwidth]{figure5.png}
\caption{Specific $[^3H]$prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) binding in the plasma membrane fraction of the neurohypophysis and serum arginine vasotocin (AVT) concentrations of laying hens at various times before and after oviposition. Samples (15 µg of protein per tube) were incubated at 30°C for 60 min with 7 nM $[^{5,6,8,9,11,12,14,15}(n)-[^3H]$PGF$_{2\alpha}$, $[^3H]$PGF$_{2\alpha}$) in the absence or presence of 35 µM unlabeled PGF$_{2\alpha}$, and the specific $[^3H]$PGF$_{2\alpha}$ binding was measured. In nonlaying hens, ranges of mean values of the specific $[^3H]$PGF$_{2\alpha}$ binding and the AVT concentration were within 394 to 435 fmol/mg of protein and 1.0 to 7.6 pM, respectively, and neither the specific $[^3H]$PGF$_{2\alpha}$ binding nor the AVT concentration significantly changed during a 24-h day. The amount of protein in membrane fractions, expressed as micrograms per milligram of wet tissue weight, was 25.7 ± 3.1 (mean ± SEM, n = 36) in laying hens and 25.2 ± 2.4 (n = 24) in nonlaying hens, respectively, and was not significantly different among the hens at different times. Each point represents the mean of 4 separate pools of samples, and the vertical bars represent SEM. *Significantly different ($P < 0.01$) from the preceding value by Newman-Keuls' test. −0 h = within 15 min before expected oviposition; 0 h = within 1 min after oviposition.}
\end{figure}
of a receptor; therefore, the presence of a PGF$_{2\alpha}$ receptor in the hen neurohypophysis is suggested.

Although the $K_d$ value was not significantly different between laying hens and nonlaying hens, the $B_{max}$ value was smaller in laying hens than in nonlaying hens (Table 2). The smaller $B_{max}$ in laying hens may be due to a difference in the secretory amount of E$_2$ and P$_4$ from the ovary, but not of testosterone, which may be suggested from the result that an administration of the E$_2$ or P$_4$ into nonlaying hens decreased the amount of specific binding of the PGF$_{2\alpha}$ receptor, but the DHT did not cause such an effect (Figure 4). The decrease in the specific binding of the PGF$_{2\alpha}$ receptor may be the result of the action of PGF$_{2\alpha}$ because the PGF$_{2\alpha}$ injection caused a decrease in the specific binding of the PGF$_{2\alpha}$ receptor (Figure 6). It has been reported that the $B_{max}$ value decreased in the AVT receptor of the shell gland (Takahashi et al., 1994) and in the chicken luteinizing hormone-releasing hormone I receptor of the anterior pituitary (Kawashima et al., 1992) after an injection of each hormone in hens. Therefore, the decrease in the amount of the specific PGF$_{2\alpha}$ receptor binding in the neurohypophysis after an injection of E$_2$ and P$_4$ may result from the stimulation of the PGF$_{2\alpha}$ production by these steroids to nerve cells of hypothalamus-neurohypophysis, which is supported by references for the production of PGF$_{2\alpha}$ in the brain containing the hypothalamus (Horton and Main, 1967; Brown and Poyser, 1984) and the presence of receptors for E$_2$ (Kawashima et al., 1987) and P$_4$ (Kawashima et al., 1978, 1979) in the hypothalamus of the hen. The E$_2$ and P$_4$ probably produce the PGF$_{2\alpha}$ in the hypothalamus and it acts on the neurohypophysis to increase the concentration of blood AVT in laying hens (Takahashi et al., 1994b).

The specific binding of the PGF$_{2\alpha}$ receptor in the neurohypophysis of laying hens showed a decrease at the time of oviposition (Figure 5), but no change was found in nonlaying hens during a 24-h period. These results suggest that the decrease in the specific binding of the PGF$_{2\alpha}$ receptor in the neurohypophysis at oviposition time of laying hens may relate to oviposition. A significant increase in the blood level of AVT with oviposition was observed when the specific binding of the PGF$_{2\alpha}$ receptor decreased (Figure 5). An injection of PGF$_{2\alpha}$ caused a decrease in the specific binding of the PGF$_{2\alpha}$ receptor and a significant increase in blood level of AVT with oviposition (Figure 6). As mentioned above, it can be thought that the PGF$_{2\alpha}$ may act in the brain as a local hormone, but the possibility of the action of blood PGF may not be denied. The PGF concentration in blood increases at spontaneous oviposition with an increase in the content of the ovarian preovulatory follicle (Saito et al., 1987; Shimada et al., 1987) and the uterus (Takahashi et al., 2004). The concentration of blood PGF is more than about 3 nM at the peak (Saito et al., 1987; Shimada et al., 1987; Takahashi et al., 1999). This is almost the same value as or higher than the $K_d$ value of PGF receptor in the neurohypophysis, obtained in the present study. The PGF$_{2\alpha}$ is transported across blood-brain barriers with facility (Bito et al., 1976). Although the origin of organ or tissue where PGF$_{2\alpha}$ is produced should be elucidated by further experiments, the present study suggests that the PGF$_{2\alpha}$ may play a direct action in the neurohypophysis on the AVT release for oviposition in hens.

**ACKNOWLEDGMENTS**

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