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Analyses for Susceptibility of Rat Anterior Pituitary Cells to Prolactin-Releasing Peptide

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We validated the effect of prolactin-releasing peptide (PrRP) on prolactin (PRL) secretion from rat anterior pituitary cells in in vitro culture. We found that culture conditions considerably influenced the response of the anterior pituitary cells to PrRP. Longer culture term (4 d) was required to obtain better responses of the anterior pituitary cells to PrRP in comparison to thyrotropin-releasing hormone (TRH). Under the culture conditions employed here, PrRP was comparable to TRH in the potency promoting PRL secretion, and the action of PrRP was very specific for PRL secretion. The susceptibility of the anterior pituitary cells to PrRP varied in female rats depending on the process of reproduction: the cells prepared from lactating rats were the most sensitive to PrRP compared with those from random-cycle and pregnant rats. Because the expression levels of PrRP receptor mRNA in the pituitary varied during the reproductive process, we speculated that the susceptibility of the anterior pituitary cells would reflect cellular changes including the expression level of PrRP receptors. In addition, treatment with estrogen in vivo enhanced the susceptibility of the cultured anterior pituitary cells in male rats. Our results indicate that the susceptibility of the rat anterior pituitary cells to PrRP is regulated by physiological mechanisms.

Key Words: Prolactin-releasing peptide; seven-transmembrane-domain receptor; prolactin; pituitary.

Introduction

Prolactin (PRL) secretion from the pituitary gland is under the complex regulation of a broad array of factors *(1)*. Although a major mechanism for regulating PRL secretion is believed to be inhibitory control by dopamine, some studies have indicated that unidentified hypothalamic factors might play important roles in the stimulatory control of PRL secretion *(1–5)*. So far many neuropeptides, e.g., thyrotropin-releasing hormone (TRH), oxytocin, vasopressin, and neurotensin, reportedly could promote PRL release in vitro or in vivo *(1)*. TRH is a representative of such factors, but its main physiological role in the pituitary is undoubtedly to stimulate the secretion of thyroid-stimulating hormone (TSH). As in the case of TRH, none of the known factors shows a stimulating activity on PRL secretion in a specific manner.

We have recently established a strategy to identify endogenous ligands for orphan seven-transmembrane-domain receptors (7TMRs) and succeeded in identifying an endogenous ligand for the orphan 7TMR, hGR3 *(6,7)*. We originally isolated hGR3 from the human pituitary and subsequently have found that its rat counterpart, UHR-1 *(8)*, was most abundantly expressed in the anterior lobe of the pituitary when examined by reverse transcriptase-polymerase chain reaction (RT-PCR) and *in situ* hybridization, although UHR-1 mRNA was also detected in other tissues including the central nervous system *(9)*. Because the endogenous ligand of hGR3 showed specific PRL release–promoting activity to the rat anterior pituitary cells in in vitro culture, we named it PRL-releasing peptide (PrRP) *(7)*.

However, Samson et al. *(10)* have recently reported that the stimulatory activity of PrRP on PRL secretion was very weak in comparison with that of TRH in in vitro experiments using primary-cultured rat anterior pituitary cells prepared from both intact male and random-cycle female rats. They are hence dubious about the physiological significance of PrRP in the regulation of PRL secretion. In this article, we report on the culture conditions under which PrRP could show a potent and specific PRL release–promoting activity to the primary-cultured rat anterior pituitary cells. After determining proper culture conditions, we compared the effects of PrRP and TRH on PRL secretion. We show here that the susceptibility of the anterior pituitary cells was changed in female rats during the reproductive process or in male rats by treatment with estrogen. In addi-

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tion, we show that the expression level of PrRP receptor mRNA was changed in the pituitary in female rats during the reproductive process and discuss the possible involvement of PrRP in the physiological regulation of PRL secretion.

Results

We first determined adequate culture conditions under which the action of PrRP on PRL secretion from primary-cultured rat anterior pituitary cells was evidently detectable. We prepared rat anterior pituitary cells according to the method reported by Vale et al. *(11)* and Shiota et al. *(12,13)* with minor modifications. We used collagenase and pancreatin for the mild dispersion of the anterior pituitary, and cultured the obtained cells in poly-D-lysine-coated plates for the efficient attachment of the cells. We used principally lactating Fischer rats as a donor for the anterior pituitaries, because these provided reproducible results in the assessment of PRL release–promoting activity in PrRP. As shown in Fig. 1, PrRP at 10^{-7} *M* slightly enhanced PRL release (i.e., about 120% compared with the control) from the rat anterior pituitary cells in the culture for 1 d. On the other hand, the effect of PrRP on PRL secretion was more evident (i.e., about 230% compared with the control) in the culture for 4 d than in that for 1 d. By contrast, the effect of TRH on PRL secretion was almost comparable (about 160–170% compared with the control) between the 1-d and 4-d culture. These results indicate that the culture term influences differently the susceptibility of the rat anterior pituitary cells to PrRP and TRH, respectively. We obtained a good response of the rat anterior pituitary cells to PrRP even after the culture for 1 wk under the conditions employed here (data not shown). Based on these results, we decided to culture the cells for 4 d to elucidate the action of PrRP on PRL secretion.

Under the same culture conditions (i.e., culture term for 4 d), we examined the effect of PrRP on the release of other pituitary hormones. As shown in Fig. 2, PrRP did not influence the secretion of growth hormone (GH), TSH, adrenocorticotropic hormone (ACTH), luteinizing hormone (LH), or follicle-stimulating hormone (FSH), even though under the same conditions growth hormone–releasing hormone (GHRH), TRH, corticotropin-releasing hormone (CRH), and luteinizing hormone–releasing hormone (LHRH) evidently promoted the secretion of these hormones, respectively. These results indicate that PrRP behaves as a specific factor for promoting PRL secretion at least in in vitro culture.

We subsequently compared the kinetics of PRL release induced by PrRP and TRH. PrRP significantly promoted PRL release within 15 min. The maximum PRL release induced by PrRP was observed between 15 min and 1 h (Fig. 3A). We therefore decided to treat the cells for 20 min to 1 h when determining the PRL release–promoting activity of PrRP in the following experiments. The increase in

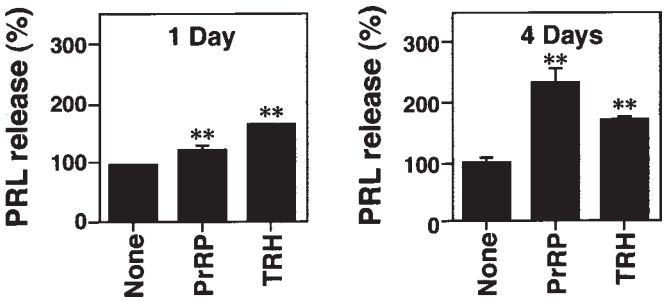

Fig. 1. Effects of culture term on PRL release from rat anterior pituitary cells by PrRP and TRH. Anterior pituitary cells prepared from lactating rats were cultured for 1 d **(left)** and 4 d **(right)**. These cells were treated with or without samples (rat PrRP31 or TRH at 10^{-7} *M*) for 1 h, and then PRL concentrations in the culture supernatants were determined. PRL concentrations in the control without the sample were 88.8 and 396 ng/mL after 1-d and 4-d culture, respectively. Data are expressed as mean values ± SEM of percentages in PRL release with each sample in comparison with the control in multiple assays ($n = 4$), except for the control of 1-d culture ($n = 3$). ** $p < 0.01$; Student's *t*-test, when compared with the control.

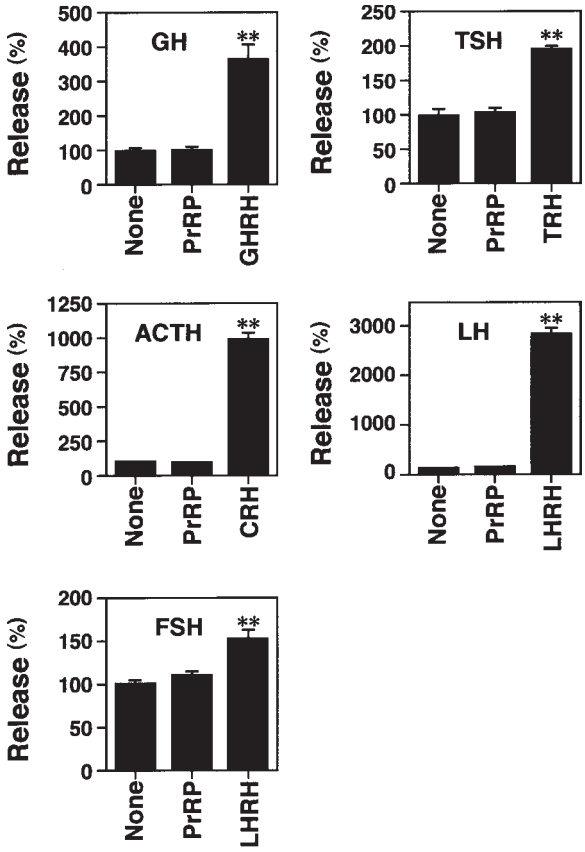

Fig. 2. Effects of PrRP on other anterior pituitary hormone secretions. Anterior pituitary cells were prepared from lactating rats and cultured for 4 d. Culture supernatants were then collected 3 h after adding the indicated peptide samples at 10^{-7} *M* except for GHRH at 10^{-9} *M*. Bovine and rat PrRP31 were used in the analyses for TSH and FSH secretions and GH, LH, and ACTH secretions, respectively. Data are expressed as mean values ± SEM of percentages in release with each sample in comparison with the control in multiple assays ($n = 3$ to 4). ** $p < 0.01$; Student's *t*-test, when compared with the control.

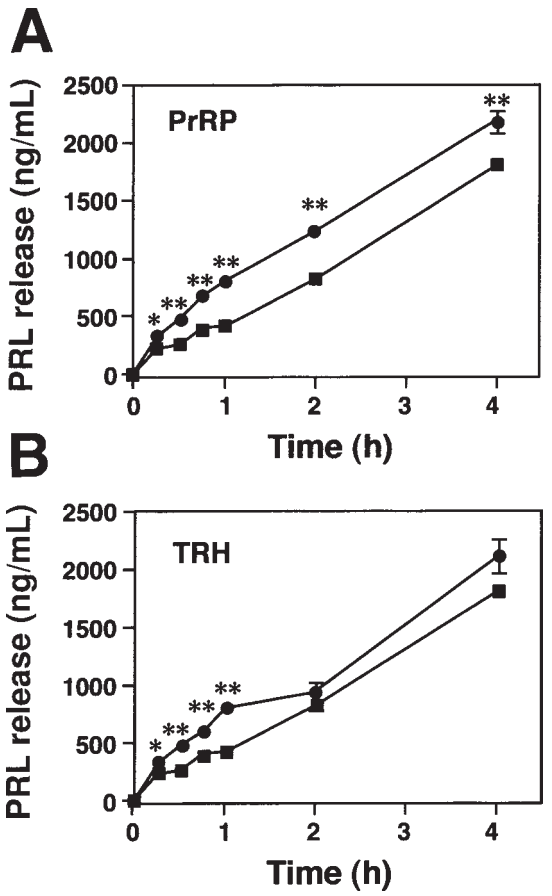

Fig. 3. Kinetics of PRL secretion from rat anterior pituitary cells after stimulation with PrRP **(A)** or TRH **(B)**. Anterior pituitary cells prepared from lactating rats were cultured under the same conditions as in Fig. 2. After the culture without (j) or with (d : bovine PrRP31 or TRH) a sample at 10^{-6} *M*, supernatants were harvested at the time indicated, and then their PRL concentrations were determined. Data are expressed as mean values ± SEM nanograms/milliliter in PRL release with each sample in comparison with the control without samples in multiple assays ($n = 4$). *$p < 0.05$; ** $p < 0.01$; Student's *t*-test, when compared with the control.

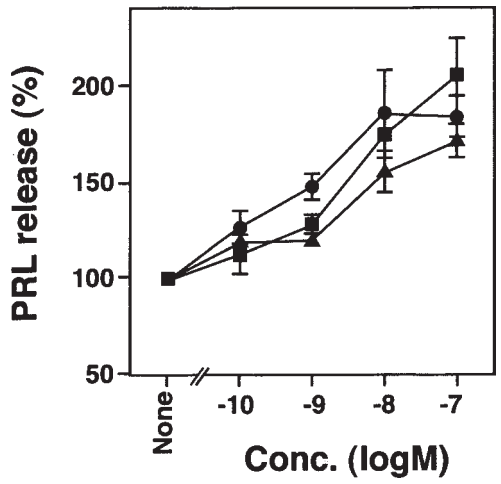

Fig. 4. Comparison of PRL secretion-stimulatory activities of bovine, human, and rat PrRPs. Anterior pituitary cells prepared from lactating rats were cultured under the same conditions as in Fig. 2. After the cells were treated with or without bovine (m), human (d), and rat (j) PrRP31 at the indicated concentrations for 1 h, PRL concentrations in the culture supernatants were determined. Data are expressed as mean values ± SEM of percentages in PRL release with each sample in comparison with the control in multiple assays ($n = 4$).

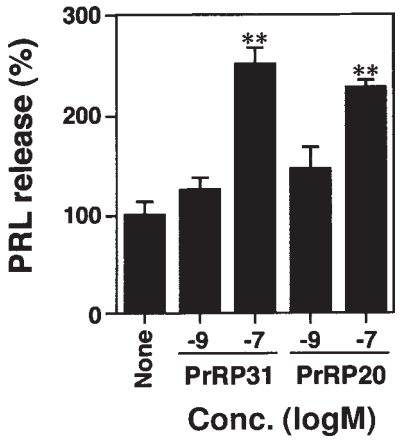

Fig. 5. Comparison of PRL secretion-stimulatory activities of PrRP31 and PrRP20. Anterior pituitary cells prepared from lactating rats were cultured under the same conditions as in Fig. 2. After the cells were treated with or without bovine PrRP31 and PrRP20 at the indicated concentrations for 20 min, PRL concentrations in the culture supernatants were determined. Data are expressed as mean values ± SEM of percentages in PRL release with each sample in comparison with the control in multiple assays ($n = 3$). ** $p < 0.01$; Student's *t*-test, when compared with the control.

PRL release induced by TRH also reached the maximum around 1 h under the same experimental conditions (Fig. 3B). We examined whether or not there were differences in the effects of bovine, human, and rat PrRPs on PRL release from the rat anterior pituitary cells. PrRP31 derived from different species showed similar potency in the promotion of PRL secretion (Fig. 4). There were no apparent differences in the potency between PrRP31 and PrRP20 (Fig. 5).

To analyze whether or not the response of the anterior pituitary cells to PrRP would change during reproductive stages, we prepared the cells from random-cycle, pregnant, and lactating female rats, respectively, and examined their responses to PrRP. As shown in Fig. 6, PRL release promoted by PrRP at both 10^{-8} and 10^{-7} *M* was significantly greater in the anterior pituitary cells prepared from lactating rats than in those from random-cycle or pregnant female rats. We repeated similar experiments twice, and obtained replicated results (Exp.1 and 2). These results indicate that the susceptibility of the rat anterior pituitary cells to PrRP is enhanced in lactating rats.

We examined the expression levels of PrRP receptor (i.e., UHR-1) mRNA in the reproductive process by means of quantitative RT-PCR as described previously *(9)*. As

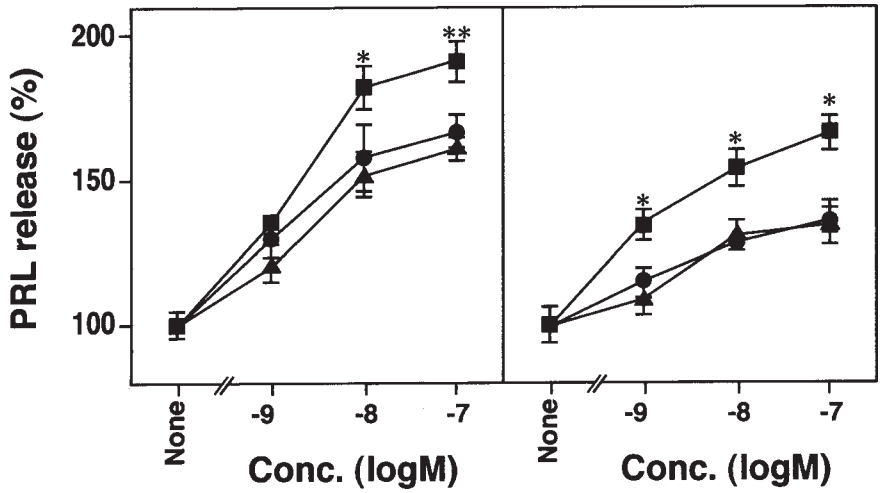

Fig. 6. Responses to PrRP in anterior pituitary cells prepared from female rats at different reproductive stages. Anterior pituitary cells were prepared from female rats of random cycle (m), 19 d in pregnancy (d), or 11 d in lactation (j), respectively. Culture supernatants were collected 1 h after adding the indicated concentrations of rat PrRP31. Experiment 1 **(left)**: PRL concentrations of the control without the sample were 439, 359, and 317 ng/mL in random-cycle, pregnant, and lactating rats, respectively. Data are expressed as mean values ± SEM of percentages in PRL release with each sample in comparison with the control in multiple assays ($n = 6$), except for the controls ($n = 12$). Experiment 2 **(right)**: PRL concentrations of the control without the sample were 492, 409, and 412 ng/mL in random-cycle, pregnant, and lactating rats, respectively. Data are expressed as mean values ± SEM of percentages in PRL release with each sample in comparison with the control in multiple assays ($n = 3$), except for the controls ($n = 6$). * $p < 0.05$; ** $p < 0.01$; Student's *t*-test, when compared to the release in cells from random-cycle rats.

shown in Fig. 7A, the expression level of PrRP receptor mRNA increased in the pituitary in pregnancy and lactation. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA, which we examined as an internal control, also increased in the same periods (Fig. 7B). In pregnancy, the increase in PrRP mRNA appeared to be greater than that of G3PDH, but the ratio between the two mRNAs in lactating rats returned to the comparable level of that in random-cycle rats. On the other hand, the yield of cells obtained from the pituitary did not increase as drastically as PrRP and G3PDH mRNAs did (i.e., 4.8×10^6, 5.1×10^6, and 5.5×10^6 cells/pituitary in random-cycle, pregnant, and lactating female rats, respectively). However, that of poly(A)$^+$ RNA apparently increased during pregnancy and lactation (i.e., 0.43, 0.79, and 1.48 μg/pituitary in random-cycled, pregnant, and lactating rats, respectively), suggesting that the content of PrRP or G3PDH mRNA increases in each cell level. These results indicate that PrRP receptors in the pituitary change considerably in cellular levels at each reproductive stage.

It is well known that treatment with estrogen in vivo enhances the plasma PRL level, accompanied by an increase in the number of lactotrophs *(1,14–16)*. We therefore examined the effect of treatment with estrogen on the response of the anterior pituitary cells to PrRP. We administered β-estradiol 3-benzoate to male rats 3 d before the assay and examined the effect of PrRP on the PRL release after a 4-d culture of the cells (Fig. 8). PrRP slightly promoted PRL release (i.e., about 120–130% compared with the control) from the anterior pituitary cells prepared from untreated male rats. On the other hand, the PRL release of the rat anterior pituitary cells prepared from the estrogen-treated rats was enhanced to about 150–175% by PrRP, indicating that the estrogen treatment increased the susceptibility of the anterior pituitary cells to PrRP.

Discussion

Because culture conditions considerably influenced the responses of the primary-cultured rat anterior pituitary cells to PrRP, we determined the proper conditions under which PrRP could exhibit an ample PRL release–promoting activity. Under the conditions we employed here, PrRP showed comparable activity to TRH in the promotion of PRL release in in vitro culture. We could detect the promotion of PRL release by PrRP using anterior pituitary cells prepared from normal male and random-cycle, pregnant, and lactating female rats, but those prepared from lactating rats were the most sensitive to PrRP.

On the other hand, Samson et al. *(10)* have reported that PrRP was less potent in PRL release–promoting activity than TRH under their culture conditions. We speculate that their culture conditions might result in reduced responsiveness of the rat anterior pituitary cells to PrRP. Several studies indicate that the procedure to disperse anterior pituitary cells sometimes considerably influences their functions in culture *(11,17–19)*. Although Samson et al. *(10)* have employed trypsin to prepare a cell suspension of rat anterior pituitary cells, Nakano et al. *(19)* have reported that the dispersion of the rat anterior pituitary cells with trypsin sometimes seriously affects the cell's hormone secretions including PRL and concluded that collagenase

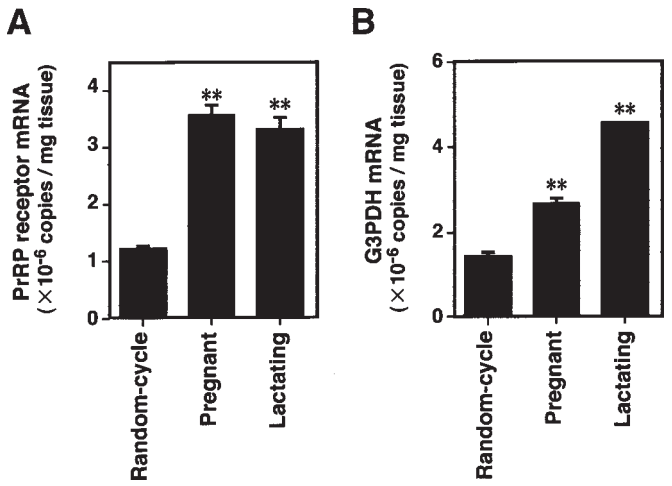

Fig. 7. Quantitative analyses for the expression levels of PrRP receptor mRNA in rat pituitaries in reproductive stages. **(A)** Quantification of PrRP receptor mRNA; **(B)** quantification of G3PDH mRNA. Pituitaries from random-cycle, pregnant, and lactating female rats were pooled, respectively, and then poly(A)$^+$ RNA prepared from them was subjected to the quantification of PrRP receptor (UHR-1) or G3PDH (internal control) mRNA expression levels as described previously *(9)*. Data are expressed as mean values ± SEM of copy number per milligram of tissue in quadruplicate analyses. ** $p < 0.01$; Student's *t*-test, when compared to the expression level in random-cycle rats.

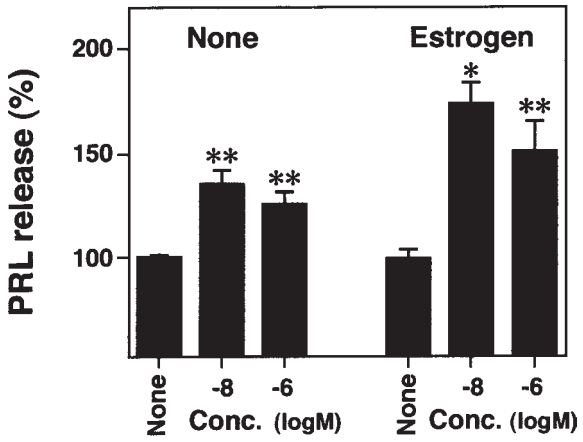

Fig. 8. Responses to PrRP in anterior pituitary cells prepared from male rats treated with estrogen. Anterior pituitary cells were prepared from male rats with or without administering β-estradiol 3-benzoate 3 d before the assay. They were cultured for 4 d, and then culture supernatants were collected 1 h after adding bovine PrRP31 at the indicated concentrations. PRL concentrations of the control without the sample were 166 and 187 ng/mL in untreated and estrogen-treated rats, respectively. Data are expressed as mean values ± SEM of percentages in PRL release with each sample in comparison with the control in multiple assays ($n = 4$). * $p < 0.05$; ** $p < 0.01$; Student's *t*-test, when compared with the control.

treatment followed by a 4-d culture would be suitable to analyze PRL secretion. Shiota et al. *(12,13)* have established the procedure to mildly disperse the anterior pituitary cells using collagenase and pancreatin, as a minor modification of the method reported by Vale et al. *(11)*.

On the basis of these studies, we used a combination of collagenase and pancreatin, which was thought to be milder for the cells in the dispersion. It was intriguing that culture term considerably influenced the susceptibility of the rat anterior pituitary cells to PrRP. Although the short-term culture for 1 d was enough to detect PRL release induced by TRH, the longer culture term for 4 d was required to obtain a better response to PrRP. These results indicate that rat anterior pituitary cells responsive to PrRP or PrRP receptor itself may be more sensitive to certain enzymatic treatments than those to TRH or TRH receptor, and adequate culture conditions are essential to provide the cells for better responses to PrRP.

In the reproductive stages, plasma PRL levels begin to increase after parturition and are high during lactation in rats. The susceptibility of the anterior pituitary cells to TRH in PRL secretion is reportedly enhanced by suckling stimuli *(20,21)*. In our study, the anterior pituitary cells prepared from lactating rats were more sensitive to PrRP in PRL secretion than those from random-cycle and pregnant rats. We found that the expression of PrRP receptor mRNA in the pituitary rose during pregnancy and lactation. Because lactotrophs increase in the pituitary during pregnancy and lactation, the increase in PrRP receptor mRNA appears to parallel that of lactotrophs. However, note that the cells prepared from pregnant rats were not as susceptible to PrRP as those from lactating rats. Therefore, the expression level of PrRP receptor mRNA does not seem to regulate solely the susceptibility of the anterior pituitary cells to PrRP. Binding experiments using a proper labeled ligand would give more information about the expression level of PrRP receptors. Further studies will be required to reveal the mechanism responsible for the enhanced susceptibility of the anterior pituitary cells obtained from lactating rats. In addition, we found that the expression level of PrRP mRNA increased in the medulla oblongata during pregnancy (unpublished data). The expression levels of both PrRP and its receptor changed during pregnancy and lactation, suggesting that they play a certain role in the reproductive process.

Although PrRP significantly promoted PRL release from the anterior pituitary cells prepared from both male and nonlactating female rats, its effect was marginal in these cases. However, the in vivo administration of estrogen afforded better responses to PrRP in male rats. We have observed that the in vivo administration of PrRP caused a specific increase in PRL in plasma in rats, although higher doses of PrRP were required to increase PRL levels in comparison with TRH *(22)*. The in vivo effect of PrRP on PRL release was considerably changed by the estrous cycle and sex in rats; that is, female rats especially in proestrus and estrus were more sensitive to PrRP than male rats *(22)*. Recently, it was reported that treatment with estrogen obviously enhanced the effect of PrRP on the elevation of

plasma PRL levels in vivo *(23)*. These facts suggest that the susceptibility of the rat anterior pituitary cells to PrRP is regulated by physiological mechanisms including estrogen. Alternatively, PrRP would work as a potent PRL-releasing factor under certain physiological conditions.

In comparison between PrRP and TRH, tissue distribution of their mRNA was quite different in the rat brain; that is, the highest expression of PrRP mRNA was observed in the medulla oblongata, whereas that of TRH mRNA was in the hypothalamus *(9)*. It has also been noted that the distribution of immunoreactive PrRP is distinct from that of the known hypothalamic hormones in the rat brain *(24,25)*. In the immunohistochemical analyses, we could not detect an apparent projection of PrRP-positive neurons to the median eminence, where the known hypothalamic hormone neurons are projected in order to act on the pituitary via the portal vessels *(26,27)*. We therefore consider that the mechanism for PrRP acting on the pituitary would differ from those for the known hypothalamic hormones. For example, Matsumoto et al. *(24)* have reported that PrRP is detected in the adrenal or posterior pituitary at relatively high levels in rats. PrRP present in these organs might be delivered to the anterior pituitary. Iijima et al. *(26)* have proposed the possibility that PrRP produced in the brain might be delivered into the systemic circulation and the cerebrospinal fluid because PrRP-positive neurons are projected to ependymal cells adjacent to the ventricle or capillary blood vessels.

Our recent studies suggest that PrRP takes part not only in the regulation of various hormone secretions including oxytocin as well as PRL *(27)*, but also in neuronal transmission or modulation *(26)*. We believe that further studies on PrRP and its receptor will provide clues that will help reveal novel mechanisms for the regulation of the endocrine and central nervous systems.

Materials and Methods

Animals

Adult male (10 wk old) or female (12–14 wk old) Fischer 344/N rats (SLC Japan, Shizuoka, Japan) were housed under controlled temperature and lighting conditions (lights on from 8:00AM to 8:00 PM) and were supplied with water and food ad libitum. All experiments using rats were conducted according to a guideline of the internal animal care and use committee. Pregnant and lactating female rats were used 19 d in pregnancy and 10–12 d postpartum, respectively.To examine the effects of estrogen, 50 μg of β-estradiol 3-benzoate (Wako, Osaka, Japan) was subcutaneously injected into the dorsal region of the neck of male rats, and the rats were housed for 3 days before use.

Culture of Anterior Pituitary Cells

Anterior pituitary cells were prepared according to the method reported by Vale et al. *(11)* and Shiota et al. *(12,13)* with minor modifications After the rats were decapitated, the anterior pituitaries were quickly removed and placed in an ice-cold buffer A consisting of 137 m*M* NaCl (Wako), 5 m*M* KCl (Wako), 0.7 m*M* Na_2HPO_4 (Wako), 25 m*M* HEPES (Dojindo, Kumamoto, Japan) at pH 7.3, 50 μg/mL of gentamicin (Gibco-BRL, Grand Island, NY), 50 U/mL of penicillin (Banyu Pharmaceutical, Tokyo, Japan), and 50 μg/mL of streptomycin (Meiji Seika Kaisha, Tokyo, Japan). They were quartered with scissors and then washed with the same buffer. The resultant dissected tissues were incubated at 37°C for 1–1.5 h in a buffer A containing 0.4% collagenase (Boehringer Mannheim, Mannheim, Germany), 10 μg/mL of DNase (Sigma, St. Louis, MO), 0.4% bovine serum albumin (BSA) (Sigma), and 0.2% glucose (Wako). The pituitary cells were dispersed by gentle trituration using a plastic pipet, and centrifuged at 320*g* for 10 min. The cell pellet was subsequently suspended in a buffer A containing 0.25% pancreatin (Sigma), and then the suspension was incubated at 37°C for 8 min. After adding fetal bovine serum (FBS) (BioWhittaker, Walkersville, MD) to stop the enzymatic digestion, the suspension was centrifuged at 220*g* for 6 min.

The cell pellets were suspended in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) containing 3.7 g/L of $NaHCO_3$, 10% FBS, 20 m*M* HEPES at pH 7.3, 50 U/mL penicillin, and 50 μg/mL of streptomycin (DMEM-I), and then filtrated through a nylon mesh (cell strainer; Becton Dickinson, Bedford, MA) to remove undigested tissue blocks. A typical yield was 1.5–5.5 × 10^6 cells/pituitary in female rats and 1.4–2.0 × 10^6 cells/pituitary in male rats. Viability of the cells determined with a trypan blue solution (Gibco-BRL) was usually more than 90%. After being washed twice with DMEM-I, the cells were cultured at 1.5 × 10^5 cells in analyses for PRL and GH secretions or at 5.0 × 10^5 cells in those for TSH, LH, FSH, and ACTH secretions in 1 mL of DMEM-I in poly-D-lysine-coated 24-well plates (Becton Dickinson) at 37°C under humidified atmosphere of 5% CO_2 in air. The culture medium in the wells was replaced every 3 d with fresh medium.

Synthetic Peptides

Bovine, human, and rat PrRP31 or PrRP20 were synthesized as described previously *(7,28)*. TRH, human GHRH and LHRH, and ovine CRH were purchased from Peptide Institute (Osaka, Japan).

Analyses for Release of Pituitary Hormones Induced by Peptides

After the anterior pituitary cells were cultured for 1 d or 4 d, they were washed three times with DMEM-II (DMEM containing 0.2% BSA and 20 m*M* HEPES at pH 7.3), and then incubated in 1 mL of the same medium at 37°C for 15 min to 1 h. After being washed twice, the cells were incubated with a sample dissolved in 1 mL of the medium. After incubation for 1 h or the time desired, culture

supernatants were harvested, centrifuged, and then stored at –30°C until used. Concentrations of PRL, GH, TSH, FSH, and LH were determined using Biotrak RIA assay systems (Amersham, Buckinghamshire, England). The concentration of ACTH was determined using a DPC·ACTH kit (Diagnostic Products, LA).

Quantitative Analyses for Rat PrRP Receptor mRNA by RT-PCR

The expression levels of UHR-1 mRNA were determined as described previously *(9)*. We prepared poly(A)$^+$ RNAs from pituitaries of random-cycle, pregnant, and lactating female rats by Isogen (Wako) and Micro-FastTrack (Invitrogen, San Diego, CA). We subsequently synthesized cDNA from each poly(A)$^+$ RNA (160 ng) treated with deoxyribonuclease I (amplification grade; Gibco-BRL) in the presence of 2.5 μ*M* random hexanucleotides (Takara Shuzo, Kyoto, Japan) and 10 U of AMV reverse transcriptase XL (Life Sciences, Petersburg, FL) at 42°C for 30 min. The primer pairs 5'-CCTGCTGGCCATTCTCCTGTCTTAC-3' and 5'-GGGTCCAGGTCCCGCAGAAGGTTGA-3' were used for the amplification to yield a 204-bp length of the PCR products. Primers to detect rat G3PDH were purchased from Clontech (Palo Alto, CA). PCR was carried out in a 25-μL reaction mixture containing 4 μL of diluted cDNA solution, 0.5 m*M* dNTPs, 5 pmol of each primer, and 0.5 μLof KlenTaq polymerase mix (Clontech). To amplify the UHR-1 cDNA, PCR was performed for 26 cycles at 98°C for 10 s and 68°C for 25 s. A 5-μL aliquot of each PCR product was electrophoresed on a 4% agarose gel and then stained with ethidium bromide. After gel images had been captured by a FOTO/Ecripse (Fotodyne, Hartland, WI) with a charge-coupled device camera, the intensity of the ethidium bromide luminescence was analyzed by a densitometry program (1-D Basic; Advanced American Biotechnology, Fullerton, CA). To obtain a calibration curve, we measured the known amounts of plasmid containing UHR-1 on the basis of optical density and then subjected it to PCR. The resultant PCR products were analyzed in the same manner as just above. No band was detected after PCR in the samples that had not been subjected to reverse transcription.

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