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Effects of Two Kinds of Chicken Luteinizing Hormone–Releasing Hormone (LH-RH), Mammalian LH-RH and Its Analogs on the Release of LH and FSH in Japanese Quail and Chicken

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A newly isolated and characterized chicken luteinizing hormone-releasing hormone-II (chicken LH-RH-II, Miyamoto *et al.*, 1984) had luteinizing hormone (LH) and follicle-stimulating hormone (FSH) releasing activity *in vitro* and *in vivo* in Japanese quail; the activity was almost equal to chicken LH-RH-I and mammalian LH-RH. These three LH-RHs induced the release of LH several times higher than that of FSH *in vitro* and also *in vivo*. No significant difference between chicken LH-RH-I and LH-RH-II was observed in LH releasing activity *in vitro* using chicken pituitary gland in the same incubating condition as in quail. Another experiment indicated that no synergism existed between chicken LH-RH-I and -II and that there was neither LH nor FSH releasing activity in [D-Phe^2, Pro^3, D-Phe^6]-LH-RH or in mesotocin. However, the same potency as in the chicken LH-RH-II was observed in [D-Ala^6, des-Gly^{10}]-LH-RH ethylamide, a superactive analog in mammals. The results indicate that an avian adenohypophysis differs from a mammalian adenohypophysis in its responsiveness to LH-RH suggesting that an avian LH-RH receptor may have a lower specificity in "recognition" of LH-RH molecules than a mammalian LH-RH receptor has.

Evidence for the existence of two kinds of gonadotropin releasing factors in the hypothalamus, one for luteinizing hormone (LH) and the other for follicle-stimulating hormone (FSH), has been provided for the first time by McCann *et al.* (1960), and Igarashi and McCann (1964), respectively. However, since the isolation of mammalian luteinizing hormone-releasing hormone (LH-RH) (Matsuo *et al.*, 1971) and the discovery of its intrinsic FSH-releasing activity, the unitary theory of gonadotropin releasing hormone is accepted more generally than the multiplicity theory.

Attempting to demonstrate the presence of FSH-releasing hormone in avian species, Igarashi and his colleagues have isolated active peptides from the chicken hypothalamus. The first substance they isolated had the following primary structure, *p*Glu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH_2 (Miyamoto *et al.*, 1982). This substance was also isolated at the same time by King and Millar (1982). However, this substance induced release of a large amount of LH and a relatively small amount of FSH from adenohypophyses of rats (Miyamoto *et al.*, 1982) and Japanese quail (Ishii *et al.*, 1984; Hattori *et al.*, 1985a) as mammalian LH-RH does in mammals. More recently, Miyamoto *et al.* (1984) isolated the second peptide whose primary structure is *p*Glu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2. Effects of this new peptide on the rat pituitary were reported by Miyamoto *et al.* (1984). However, no study has been published for the effect of this peptide on the avian adenohypophysis except two brief papers (Ishii *et al.*, 1984; Millar and King, 1984).

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The present paper reports the effects of two kinds of chicken LH-RHs as well as mammalian LH-RH *in vivo* and *in vitro*. To consider the specificity of the responsiveness of the avian adenohypophysis to LH-RHs, two analogs of mammalian LH-RH and mesotocin were also tested by our *in vitro* method.

MATERIALS AND METHODS

Animals. Three-week-old male Japanese quail (*Coturnix coturnix japonica*) were obtained from a commercial source. They were kept under a short day length (8 hr light:16 hr darkness) and served as adenohypophysial donors between 6 and 10 weeks of age. Before the start of experiments, the cloacal region of each bird was examined, and only individuals with the completely regressed cloacal protrusion were selected for the experiments. Young cockerels were obtained before sexual maturation from a broiler company and served as adenohypophysial donors on the same day.

In vitro experiments. Adenohypophyses were removed immediately after decapitation and cut midsagittally into two halves. Sixteen adenohypophysial halves were put into 16 small stainless-steel baskets which were hung with thin wires from a frame in a row (Fig. 1). Three rows of 16 polypropylene incubation tubes each containing 1 ml of medium 199, pH 7.4, were prepared. Into the tubes in the third row, 50 μl of various concentrations of peptides in saline or saline alone was added. The peptides were chicken LH-RH-I (supplied by Professor Yanaihara), chicken LH-RH-II (supplied by Professor Igarashi), mammalian LH-RH (NIAMDD), [D-Ala^6, des-Gly^{10}]-LH-RH ethylamide (Peninsula Laboratories, Inc.), [D-Phe^2, Pro^3, D-Phe^6]-LH-RH (Peninsula Laboratories, Inc.), and mesotocin (Bachem Inc.). The baskets containing the glands were dipped into the medium of the first row of tubes and preincubated at 37° for 3 hr under atmosphere of 95% O_2 and 5% CO_2 with continuous shaking. Then, the baskets were transferred successively to the second and third rows and similarly incubated for 10 min at each row. The media in the second row were used as the sample for the initial levels and those in the third row for the induced levels by LH-RHs and mesotocin. The experiment was performed in five to eight replicates for each peptide and all the incubation media were stored at −20° until assayed for LH and FSH.

In vivo experiments. Chicken LH-RH-I, -II, and mammalian LH-RH were used for *in vivo* experiment. Fifty microliters of the LH-RH solution containing 0.2, 1, or 5 μg of the substance or saline was rapidly injected (within 3 sec) into the right jugular vein of the quail. Blood samples were collected into heparinized syringes from a brachial wing vein just before and at 2, 5, and 10 min after the injection. Since LH and FSH levels peaked at 5 min and were decreased at 15 min after a single injection of LH-RH as is shown in our previous paper (Hattori *et al.*, 1985a), we collected blood samples only for the first 10 min to cover a peak of the changes. The plasma was separated by centrifugation and stored at −20° until assayed for LH and FSH.

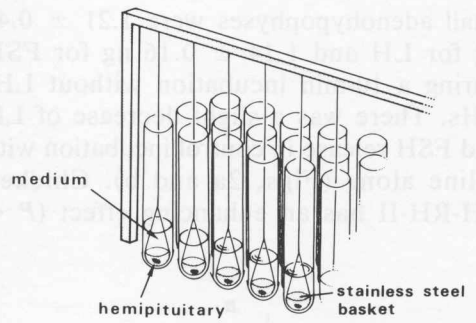

FIG. 1. An apparatus for studying the effects of LH-RH in releasing LH and FSH from the adenohypophysis *in vitro*. Adenohypophysial halves were placed in stainless-steel baskets suspended by thin wires from the frame; the baskets were dipped into incubation tubes containing medium 199. The baskets were arranged in rows so that 16 halves could be tested by simultaneous transfer to a new row of tubes.

Radioimmunoassay of gonadotropins. The LH and FSH concentrations in samples were determined by the method of Hattori and Wakabayashi (1979) for LH and by that of Sakai and Ishii (1983) for FSH with slight modifications. In the present assay system, the sample volume was reduced to 50 μl for the incubation medium sample and 25 μl for the plasma sample. The minimum detectable level of LH assay was 5 pg per tube and the intra- and interassay coefficients of variation were 2.8 and 12.2%, respectively. The minimum detectable level of FSH assay was 21 pg per tube and the intra- and interassay coefficients of variation were 4.8 and 5.8%, respectively. Determination of the samples was made in duplicate. Results are expressed in terms of the most highly purified preparation of chicken LH (fraction IRC-2, Gunma) and chicken FSH (fraction AGCHDS111135A).

Statistical analysis. Results were analyzed for significance by Duncan's multiple range test, and by one-way or two-way layout analyses of variances.

RESULTS

LH and FSH Release from Quail Adenohypophyses in Response to Varying Concentrations of Chicken LH-RH-I, -II, and Mammalian LH-RH in Vitro

The initial LH and FSH releases from

quail adenohypophyses were 1.21 ± 0.45 ng for LH and 1.14 ± 0.16 ng for FSH during a 10-min incubation without LH-RHs. There was a slight decrease of LH and FSH release in control incubation with saline alone (Figs. 2a and b). Chicken LH-RH-II has an enhancing effect ($P < 0.01$) on the release of LH and FSH from quail adenohypophyses (Figs. 2a and b). The maximum LH release from the glands treated with 50 ng of chicken LH-RH-II per ml was 17 times that of the release in control and the maximum FSH release was 4.3 times at the same dose. At 250 ng/ml, how-

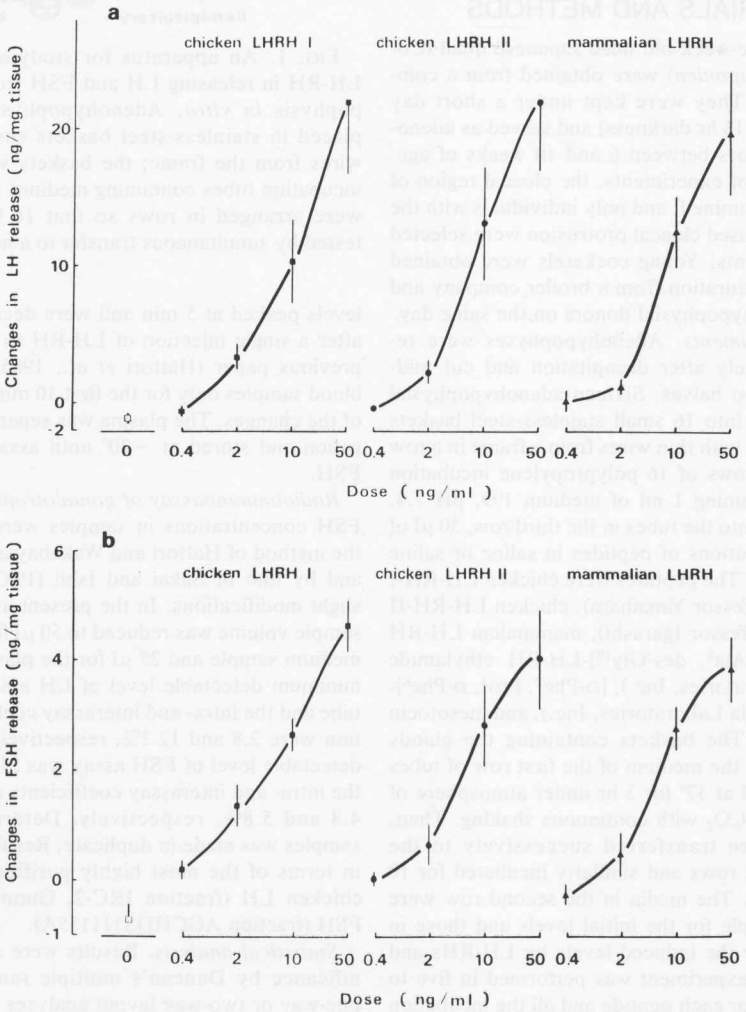

FIG. 2. Changes in the release of LH (a) and FSH (b) from a half of the quail adenohypophysis *in vitro* in the presence of different concentrations of chicken LH-RH-I (closed squares), LH-RH-II (circles), or mammalian LH-RH (triangles). Doses of LH-RHs (ng/ml) are shown on the horizontal axis in a logarithmic scale. The vertical axis shows the changes in LH and FSH release from the initial levels. The saline control group (open square) is indicated in the left part of each figure. Each point represents the mean ± SEM of LH and FSH release in six replicates. Note the difference in the vertical axis scale between (a) and (b).

ever, no further increase was observed in either the LH or FSH release (data not shown).

Chicken LH-RH-I and mammalian LH-RH also increased the LH and FSH releases ($P < 0.01$) at about the same rate as with chicken LH-RH-II (Figs. 2a and b). We calculated the relative potency between chicken LH-RH-I, -II, and mammalian LH-RH using a 2 × 3-point design of a parallel-line assay. There was no significant difference in LH- and FSH-releasing potencies among three LH-RHs ($P > 0.05$). The mean potency ratios to chicken LH-RH-I were 1.1 for chicken LH-RH-II and 0.84 for mammalian LH-RH in the LH release, and 0.82 and 0.71 in the FSH release, respectively.

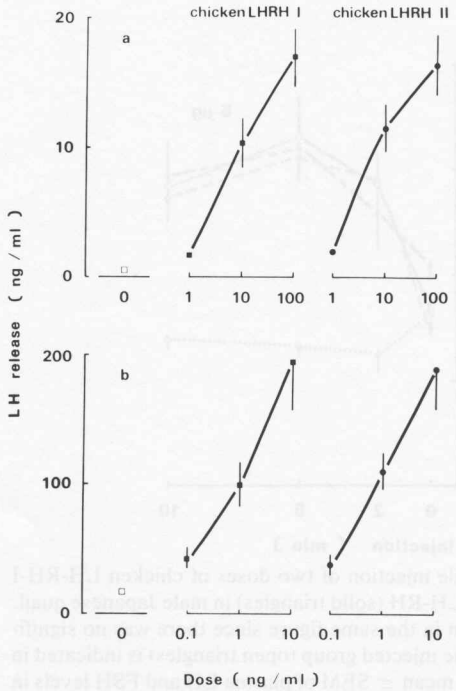

FIG. 3. Release of LH from adenohypophysial halves of Japanese quail (a) and chicken (b) *in vitro* in the presence of different concentrations of chicken LH-RH-I (closed squares) or chicken LH-RH-II (circles). Doses of LH-RHs (ng/ml) are shown on the horizontal axis in a logarithmic scale, and the LH release on the vertical axis. The saline control level (open square) is indicated in the left part of each figure. Each point represents the mean ± SEM of LH release in five to seven replicates. Note the difference in scales for the vertical and horizontal axis between (a) and (b).

Comparison of LH Release from Adenohypophyses of Quail and Chicken in Vitro between the Response to Chicken LH-RH-I and Chicken LH-RH-II

In this experiment, one-half of each adenohypophysis was exposed to chicken LH-RH-I while the contralateral half was exposed to chicken LH-RH-II. The one half was distributed randomly whether right or left.

Both chicken LH-RH-I and -II significantly ($P < 0.01$) increased the release of LH from quail and also chicken adenohypophyses in a dose-dependent manner (Figs. 3a and b). The mean potency ratio of chicken LH-RH-I to chicken LH-RH-II was 1.00 for chicken adenohypophysis and 0.99 for quail adenohypophysis in the release of LH using a 2 × 3-point design of a parallel-line assay.

Changes in Plasma FSH and LH Levels after a Single Injection of Chicken LH-RH-I, -II, or Mammalian LH-RH in Quail

Chicken LH-RH-II as well as chicken LH-RH-I and mammalian LH-RH induced significant increases ($P < 0.01$ or $P < 0.05$) in plasma LH at dose levels of 1 and 5 μg per bird even 2 min after the injection (Fig. 4a). At the dose of 0.2 μg per bird, one or two out of six individuals responded to LH-RHs by increasing plasma LH levels but the others did not. The mean concentration of LH was highest in the chicken LH-RH-I injected group among four groups 2 and 5 min after the injection of 1 μg of hormone and also 2, 5, and 10 min after the injection of 5 μg of hormone. However, the comparison of the means among three hormone-injected groups at the 1 or 5 μg dose levels by means of a two-way layout analysis of variances using

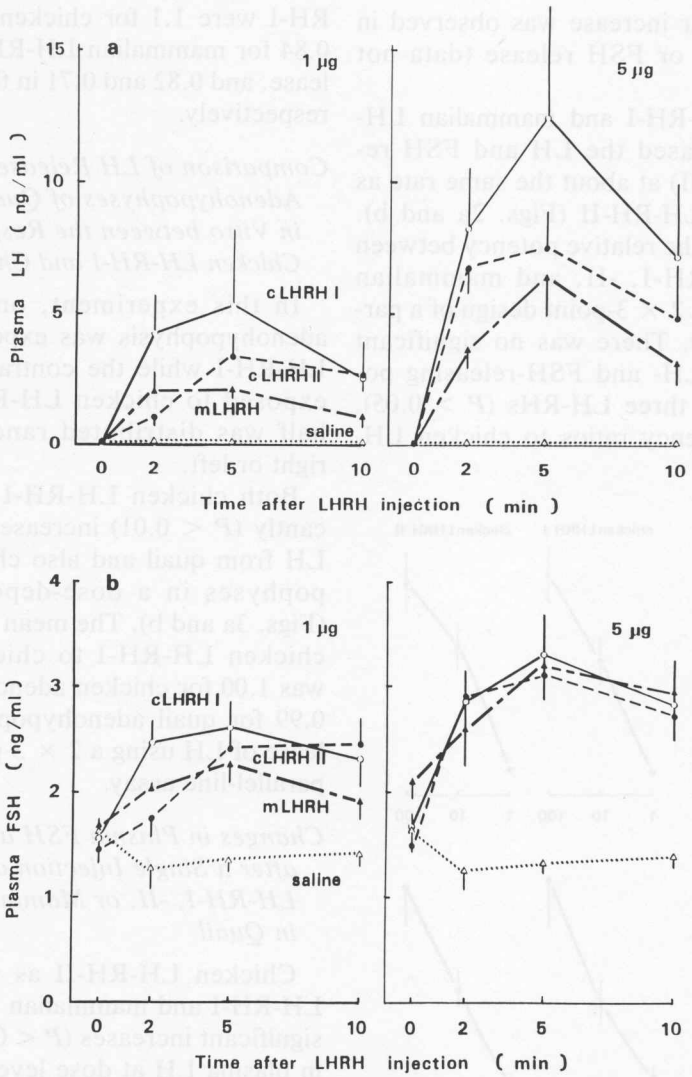

FIG. 4. Plasma LH (a) and FSH (b) following a single injection of two doses of chicken LH-RH-I (open circles), LH-RH-II (solid circles) or mammalian LH-RH (solid triangles) in male Japanese quail. The injection of 0.2 μg LH-RH per bird was not shown in the same figure since there was no significant difference in plasma gonadotropin levels. The saline injected group (open triangles) is indicated in the lower part of each figure. Each point represents the mean ± SEM of plasma LH and FSH levels in five to seven replicates. The injection of LH-RHs or saline was performed at time zero.

the log-transformed data revealed that the difference of the means among the groups was not significant ($P = 0.312$ and 0.07, respectively). Furthermore, comparisons of the means among the three hormone-treated groups at the 5 μg dose level at 5 min by means of a one-way layout analysis of variances using log-transformed data also showed that the difference was not significant. Thus, we failed to detect a significant difference for the mean LH concentration among LH-RHs at any postinjection times or at any hormone dose levels.

The mean concentrations of FSH in all the three LH-RH-injected groups behaved similarly to each other and significantly (P

< 0.01) higher than the concentration of the control group at both dose levels and at most of the postinjection times (Fig. 4b). Furthermore, there were no significant differences among LH-RH-injected groups at any dose levels or at any postinjection times, except for a single case: i.e., the difference between chicken LH-RH-I and chicken LH-RH-II at the 1 μg dose level 2 min after the injection.

Effects of Chicken LH-RH-II, Analogs of Mammalian LH-RH, Mesotocin, and Combination of Chicken LH-RH-I and -II on the Release of LH and FSH from Quail Adenohypophysis in Vitro

Quail adenohypophysial halves were treated for 10 min with 0.4, 2, 10, and 50 ng per ml of chicken LH-RH-II alone, a mixture of chicken LH-RH-I and LH-RH-II in the ratio of 1:1, [D-Ala^6, des-Gly^{10}]-LH-RH ethylamide, [D-Phe^2, Pro^3, D-Phe^6]-LH-RH, mesotocin, or saline, and changes of the LH and FSH releases were measured. Each of the following substances—chicken LH-RH-II, the mixture of chicken LH-RH-I and LH-RH-II, and [D-Ala^6, des-Gly^{10}]-LH-RH ethylamide—stimulated the release of LH (Fig. 5) and there was no significant difference in the increase of the release among three groups at any dose levels ($P > 0.05$). In contrast, neither [D-Phe^2, Pro^3, D-Phe^6]-LH-RH, mesotocin, nor saline stimulated the release of LH (Fig. 5).

The release of FSH was also stimulated by chicken LH-RH-II, the mixture of chicken LH-RH-I and -II, and [D-Ala^6, des-Gly^{10}]-LH-RH ethylamide but not by [D-Phe^2, Pro^3, D-Phe^6]-LH-RH, mesotocin or saline (Fig. 6). At the highest dose level, however, there was no further increase in the FSH release in any effective substances.

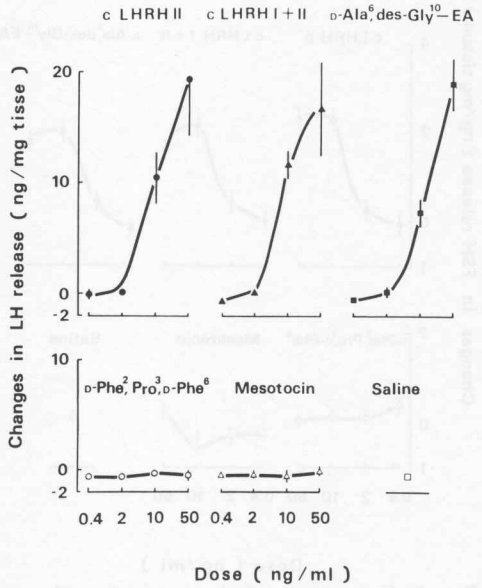

FIG. 5. Comparison of changes in the release of LH from a half of quail adenohypophysis *in vitro* in response to chicken LH-RH-II alone, a 1:1 mixture of chicken LH-RH-I and -II, analogs of mammalian LH-RH, mesotocin, and saline. Doses of hormones (ng/ml) are shown on the horizontal axis in a logarithmic scale. The vertical axis shows the changes in LH release from the initial levels. The treated hormones and saline into the medium are indicated in the upper part of each figure. Each point represents the mean ± SEM of LH release in five to eight replicates.

DISCUSSION

For chicken LH-RH-II, we were able to demonstrate (1) that this peptide can stimulate release of both FSH and LH from the adenohypophysis of Japanese quail, (2) that the increment of the FSH release induced by this peptide is smaller than that of the LH release, and (3) that no significant difference was observed in either LH- or FSH-releasing potency between chicken LH-RH-II and chicken LH-RH-I, or between chicken LH-RH-II and mammalian LH-RH. Thus, we were able to show that chicken LH-RH-II is very similar to chicken LH-RH-I and mammalian LH-RH in its biological activity in birds and that this peptide is not the releasing hormone which has solely or mainly FSH-releasing activity. These were derived from the *in vivo* experiments in which the samples were collected only for 10 min after a single injection of the LH-RHs, and from the *in*

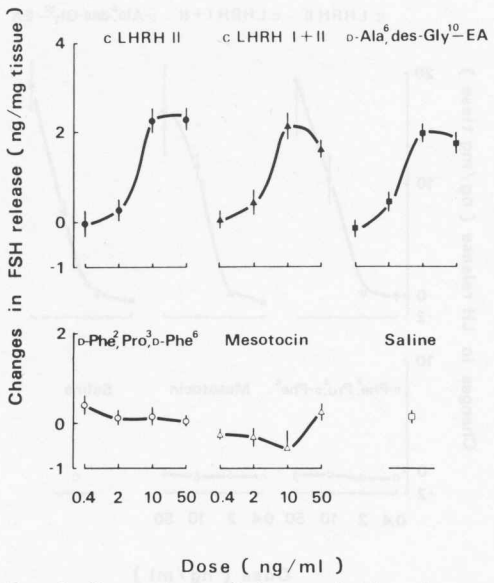

FIG. 6. Comparison of changes in the release of FSH from a half of quail adenohypophysis *in vitro* in response to chicken LH-RH-II alone, a 1:1 mixture of chicken LH-RH-I and -II, analogs of mammalian LH-RH, mesotocin, and saline. For further details, see the legend of Fig. 5 and the text.

vitro experiments in which the LH-RHs were applied for 10 min to the cultured quail adenohypophyses. Our recent results (Hattori *et al.*, 1985b) indicated that the pulse stimulation of LH-RHs for 10 min with moderate intervals stimulated LH and FSH release repeatedly, whereas prolonged application of the LH-RHs was not able to maintain the gonadotropin release and it rather induced gradual decrease of the gonadotropin release. The results seem to indicate that LH-RH exerts its action to the full when it is released in episodic manner. In this paper we, accordingly, utilized a short period incubation (Fig. 1) rather than a long period culture to examine the LH-RH effects.

In the present experiment, we used the adenohypophyses from quail maintained on short days to reduce individual variations in responsiveness to the LH-RH. This, together with a shorter incubation time, made difference in the values of initial LH and FSH release between the present experiment and our previous experiment (Hattori *et al.*, 1985a; Hattori *et al.*, 1986) in which the adenohypophysis was taken from quail on long days.

Some of these results confirm previous findings that chicken LH-RH-I is almost equipotent to mammalian LH-RH in stimulating LH release (Millar and King, 1983; Ishii *et al.*, 1984; Sterling and Sharp, 1984; Hattori *et al.*, 1985a) and in stimulating FSH release (Ishii *et al.*, 1984; Hattori *et al.*, 1985a). In rats, however, chicken LH-RH-I and chicken LH-RH-II are far less active than mammalian LH-RH (for chicken LH-RH-I, Yanaihara *et al.*, 1972; Miyamoto *et al.*, 1982, for chicken LH-RH-II, Miyamoto *et al.*, 1984). The similarity of biological potencies of chicken LH-RH-II to chicken LH-RH-I in birds, in other words the coexistence of two very similar gonadotropin-releasing hormones in the avian hypothalamus complicates our discussion of the unitary or multiplicity theory of gonadotropin-releasing hormone.

Millar and King (1984) described in their review that chicken LH-RH-II has approximately six times the potency of chicken LH-RH-I and mammalian LH-RH in stimulating LH and FSH release from dispersed chicken pituitary cells. It is likely that the discrepancy between our *in vitro* results and those of Millar and King is derived from the incubation time; they incubated for 2 hr using dispersed anterior pituitary cells. Conventional culture methods with longer incubation times do not seem to provide accurate information on the physiological characteristics of the effect of neurosecretory hormones which have self-priming and/or desensitization effects on the tissue. We have observed that chicken LH-RH-I and LH-RH-II only induced a transient release of LH even though the stimulants were applied for longer periods using our *in vitro* incubation system (Hattori *et al.*, 1985b). More recently, Sharp *et al.* (1986) reported chicken LH-RH-II was more effective than chicken LH-RH-I or mamma-

lian LH-RH, and mammalian LH-RH was slightly more active than chicken LH-RH-I in stimulating LH release by an intravenous injection to incubating bantam hens. Sterling and Sharp (1984) described in their earlier report using cockerels *in vivo* that the LH-releasing activities of mammalian LH-RH and chicken LH-RH-I are the same. Discrepancies between their former and latter reports and between their former report and our present *in vivo* results may be explained by the sex difference in responsiveness to LH-RHs; a single injection of LH-RH exerted more prolonged increase of plasma LH in mature female birds than in male birds (Sterling and Sharp, 1984).

Quite recently, the distribution of immunoreactive LH-RH-perikarya and fibers in the chicken and quail brains examined (Mikami, personal communication) using two different anti-chicken LH-RH sera (Hasegawa *et al.*, 1986): one (anti-cLH-RH-I) was raised against chicken LH-RH-I and the other (anti-cLH-RH-II) against chicken LH-RH-II, 2–10. A number of perikarya of the nucleus preopticus and the nucleus septalis were stained immunocytochemically with anti-cLH-RH-I serum. Many immunoreactive LH-RH fibers were distributed through the external layer of both anterior and posterior median eminence. However, anti-cLH-RH-II did not reveal any LH-RH perikarya of the nucleus preopticus or the nucleus septalis, but it showed a positive reaction in the lateral parts of the nucleus tuberalis and in the anterior to dorsal parts of the root of the nervus oculomotorius. Moreover, no immunopositive reaction was detectable in the median eminence by using the anti-cLH-RH-II. For the lack of LH-RH-II immunoreactivity in the median eminence, two mutually exclusive possibilities could be considered. The chicken LH-RH-II may have a very quick turnover rate and may not be stored in the median eminence. Alternatively, chicken LH-RH-II may not be released from the median eminence, in other words, chicken LH-RH-II may act not as a hormone but as a neurotransmitter or neuromodulator. In support of the latter explanation, Jan *et al.* (1979) and Eiden *et al.* (1982) have presented results suggesting that LH-RH acts as a neurotransmitter in the sympathetic ganglia and retinae of the frog.

As mentioned above, the avian adenohypophysis was not able to differentially respond to the three kinds of LH-RH, while mammalian adenohypophysis was able to discriminate clearly among them in the intensity of the response; LH-releasing potency ratios among them (mammalian LH-RH: chicken LH-RH-I: chicken LH-RH-II) being 100: 5: 32 (Yanaihara *et al.*, 1972; Miyamoto *et al.*, 1982; Miyamoto *et al.*, 1984). Furthermore, [D-Ala^6, des-Gly^{10}]-LH-RH ethylamide, which is a superactive analog of LH-RH in mammals and fish (Coy *et al.*, 1974; Van der Kraak *et al.*, 1983), was no longer a superactive analog but roughly an equipotent analog in quail in our *in vitro* incubation system. This does not imply that all analogs of LH-RH which are superactive in mammals are always equipotent in birds. Sterling and Sharp (1984) showed in chickens that [D - Ser - (Bu^t)^6 - des-Gly^{10}]-LH-RH ethylamide was about twice as potent as mammalian LH-RH in LH release. However, this analog releases 190 times as much LH as the same dose of mammalian LH-RH in rats (Hsueh *et al.*, 1983). Hasegawa *et al.* (1984) reported that [D-Leu^6, des-Gly^{10}]-LH-RH ethylamide was 26 times more active than mammalian LH-RH in stimulating LH release in rats but was slightly more active in chickens. Thus in birds, the activity of the analogs of mammalian LH-RH in stimulating LH release seems to be much less than in mammals, if they are not equipotent. On the other hand, [D-Phe^2, Pro^3, D-Phe^6]-LH-RH was as inactive in the quail adenohypophysis as it is in the mammalian adenohypophysis when it acts alone (Bowers *et al.*, 1980). These results suggest that LH-RH receptors in the avian gonado-

tropes have low specificity to various types of LH-RHs and derivatives.

It might be interesting to speculate about the relationship between the biological properties and their primary structures in LH-RHs. Studies with LH-RHs and derivatives in the mammal show that at least the His-Trp residues of position 2 and 3 are indispensable for the appearance of biological activity (see for review Schally and Coy, 1983) and the arginine at the position 8 determines affinity to the LH-RH receptor (e.g., Sandow *et al.*, 1978). As already reported by many investigators, substitution of Arg^8 with some amino acids decreases the biological potency in mammals. However, in the quail, substitution of position 8 with Gln or Tyr did not affect the biological potencies. In birds, accordingly, the position in the LH-RH molecule which is related to receptor binding affinity may be different from mammals or the recognition site of LH-RH molecules in the LH-RH receptor may be less specific than mammals. It is highly probable that the LH-RH receptors of birds are different from those of mammals in their binding property. On the other hand, the active core of the LH-RH molecule seems to be the same also in birds, since two avian LH-RHs retain the His-Trp residues at the position 2 and 3, and [D-Phe^2, Pro^3, D-Phe^6]-LH-RH, whose position 2 and 3 are substituted, was inactive in the quail as in the rat (Bowers *et al.*, 1980).

It might be too premature to venture discussion of molecular evolution of LH-RH in vertebrate species having examined the primary structure of only 5 types of LH-RH; lamprey, salmon, two types of chicken, and mammal. Even in neurohypophysial hormones, which have been far better studied than LH-RHs since 30 years ago, we have come across a number of unexpected facts; the finding of lower vertebrate hormone species in the fetus of mammals, and the detection of oxytocin in invertebrates. Accordingly, it may be safe to speculate only that chicken LH-RH-II may be an LH-RH species which has emerged rather early in the course of vertebrate evolution, since a chicken LH-RH-II-like peptide has been demonstrated in dogfish and trout brains by comparing the retention time of immunoreactive LH-RH on an isocratic HPLC system (Sherwood and Sower, 1985).

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