Different mechanisms controlling FSH and LH release in Japanese quail (*Coturnix coturnix japonica*): evidence for an inherently spontaneous release and production of FSH

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ABSTRACT

Plasma concentration of FSH and LH and their content in the adenohypophyses of Japanese quail were estimated by homologous radioimmunoassays based on chicken hormones. The results consistently showed that the plasma concentration of FSH was slightly higher than that of LH, although the FSH content in the adenohypophysis was much lower than that of LH in immature, mature and acutely photostimulated quail. These results prompted experiments *in vitro* to determine whether the difference was intrinsic. Adenohypophyses were collected and each cut mid-sagittally into two halves; one half was incubated for 20 h with or without chicken hypothalamic extract, and the medium was changed every 1 or 2 h to estimate the FSH and LH released. The other half served to estimate the initial content of FSH and LH in the adenohypophysis. The amount of FSH released was three times more than the original content even when adenohypophyses were incubated without hypothalamic extract, and the residual FSH content in the pituitary after 20 h of incubation was equal to the initial FSH content. In contrast, under the same incubation conditions, little LH was released and the residual LH content was decreased. When hypothalamic extract was added to the medium, LH release was enhanced sixfold compared with the control, whereas the increase in FSH was less than twofold. Spontaneous FSH release was markedly decreased in a Ca^{2+}-deficient incubation medium, suggesting that the release was not due to leakage but to an active secretion mechanism. We therefore suggest, for the first time, that the release and production of FSH are largely autonomous, whereas release and production of LH are rigidly controlled and regulated by the releasing hormone in avian species.

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INTRODUCTION

One of the major challenges in reproductive endocrinology has been to answer the question of how the secretion of follicle-stimulating hormone (FSH) and of luteinizing hormone (LH) are separately controlled in view of the different roles of each hormone at the gonadal level.

Most of the studies so far conducted in mammals have been designed to find out whether LH and FSH are regulated by separate agents secreted from the hypothalamus (e.g. Bowers, Currie, Johansson & Folkers, 1973) or whether a single gonadotrophin-releasing hormone controls the release of both hormones in conjunction with feedback actions of steroid hormones (e.g. Debeljuk, Vilchez Martinez, Arimura & Schally, 1974) or inhibin (e.g. Franchimont, Chari, Hagelstein & Duraiswami, 1975) to give differential release of the gonadotrophins. However, many questions are still unsolved.

There have been many studies on the mechanism of LH release in birds (Bonney & Cunningham, 1977*a*, *b*; Luck & Scanes, 1980), but few on that of FSH secretion. Using the homologous chicken FSH radioimmunoassay (RIA) of Sakai & Ishii (1983), experiments were carried out to study whether the release of FSH and LH are controlled differently. Thus, the purpose of the present study was (1) to investigate *in vivo* whether there are differences between the concentrations of FSH and LH in the circulation and in the

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content of the pituitary under different conditions, and (2) to investigate *in vitro* how any differences are derived.

MATERIALS AND METHODS

In-vivo experiments

Experiment 1

Male Japanese quail (*Coturnix coturnix japonica*) were reared from hatch under a long photoperiod of 16 h light and 8 h darkness (16L : 8D). They were killed at 4, 9 and 30 weeks of age. Blood samples were collected into heparinized tubes and the plasma was separated by centrifugation. Pituitary glands were dissected and stored frozen in phosphate-buffered saline (0·01 mol/l, pH 7·5) containing 1% bovine serum albumin at −20 °C before extracts were prepared. The pituitaries were homogenized in Teflon–glass homogenizers, centrifuged, and the supernatants stored at −20 °C until assay.

Experiment 2

Male quail were reared from hatch under short days (8L : 16D) until they were 35 days of age, when they had attained virtually full somatic growth but were reproductively immature. One group of male quail was killed at 35 days, the other group was transferred to 16L : 8D for 8 days before autopsy. Blood samples and the pituitary glands of the two groups were collected, and the plasma and the supernatants of pituitary homogenates were stored at −20 °C until assay.

In-vitro experiments

Animals

Male Japanese quail were obtained from the Suzukei Company, Toyohashi City at the age of 5 weeks. They were kept under long days of 16L : 8D for about 3 weeks before each experiment.

Preparation of extract of basal hypothalamus

Heads of fresh broiler chickens were obtained from Ryoto Broiler Company Ltd, Chiba City and kept in a container with ice until removal of the basal hypothalamus on the same day. The basal hypothalamus largely consisted of median eminence only. About 120 pieces of the tissue were pooled in 1 ml ice-cold HCl (0·1 mol/l). An equivalent weight of cerebellum was taken as a control. Tissue was homogenized, frozen and thawed, and then centrifuged at 12 000 *g* for 30 min at 4 °C. The supernatant fluid was withdrawn, and the precipitate resuspended in HCl (0·1 mol/l), centrifuged again, and this supernatant layer was combined with the original. The combined extract was neutralized to pH 7·4–7·6 with NaOH (1 mol/l), recentrifuged and the supernatant stored at −20 °C until incubation experiments were performed.

Incubation procedure

Anterior pituitary glands were removed immediately after decapitation of the quail, divided mid-sagittally into two halves under a binocular microscope and kept on ice. Each hemipituitary was weighed to the nearest 10 μg. Incubation was carried out in a metabolic shaker at 37 °C under an atmosphere of 95% O_2 and 5% CO_2 using Medium 199 (pH 7·4). In the first experiment, three hemipituitaries/flask were preincubated for 3 h and then incubated for a further 2 h with various concentrations of hypothalamic extract (tissues derived from 0·18 to 4·9 chickens/ml) or with physiological saline. At the end of the incubation, all media were stored frozen until assayed. In the second experiment, one half of each pituitary was incubated for 20 h with hypothalamic extract (tissues derived from 4·8 chickens/ml) or with cerebellum extract, while the contralateral half was used to measure the original content of FSH and LH in the hemipituitary. At intervals of 1 or 2 h, and at the end of the incubation, 500 μl samples of media were withdrawn and stored frozen for assay. After 20 h of incubation, the hemipituitary in the flask was homogenized, centrifuged and the supernatant stored frozen until the residual FSH and LH content was determined. In the third experiment, the Ca^{2+} concentration of the incubation medium was reduced to 1/6 of the original (1·8 mmol/l) with EGTA. In a further group, the original Ca^{2+} concentration was restored by adding $CaCl_2$. In the control group, the original incubation medium was used. In these three groups, hypothalamic extract was not added to the incubation media.

Radioimmunoassays

For the RIA of FSH, we followed the method of Sakai & Ishii (1983) exactly. Results are expressed in amounts of the chicken FSH fraction, AGCHDS111135A, of Sakai & Ishii (1980). Luteinizing hormone was measured by a micromodification (the sample volume being reduced to 50 μl) of the method described by Hattori & Wakabayashi (1979). Concentrations of LH are expressed as amounts of the chicken LH fraction IRC-2(Gunma) which was supplied by Professor K. Wakabayashi of Gunma University, Japan. The interassay coefficients of variation (C.V.) for FSH and LH assays were 4·9 and 10·5 respectively. Corresponding values for the intra-assay C.V. were 3·9 and 2·8.

Calculation of amounts of released hormones

Immunoreactive FSH and LH in the basal hypothalamic and cerebellum extracts were determined by specific radioimmunoassays. Amounts of FSH and LH released into the medium were estimated by subtracting the amount of immunoreactive hormones in

the tissue extracts from the total content in the medium after incubation.

Statistical analysis

Results were analysed for significance by Duncan's multiple range test or paired and unpaired *t*-test. Dose–response relations were evaluated by linear regression analysis.

RESULTS

Comparison of the content of FSH and LH in the pituitary glands and of their circulating concentrations at different ages and under different photoperiodic conditions

The content of FSH in the pituitary was much lower than that of LH, but the concentration of FSH in plasma was slightly higher than that of LH at all ages (Fig. 1). Most notably, at 4 weeks of age, FSH content in the pituitary was only 35% of that of LH, but the plasma FSH concentration was about 3·5 times that of LH.

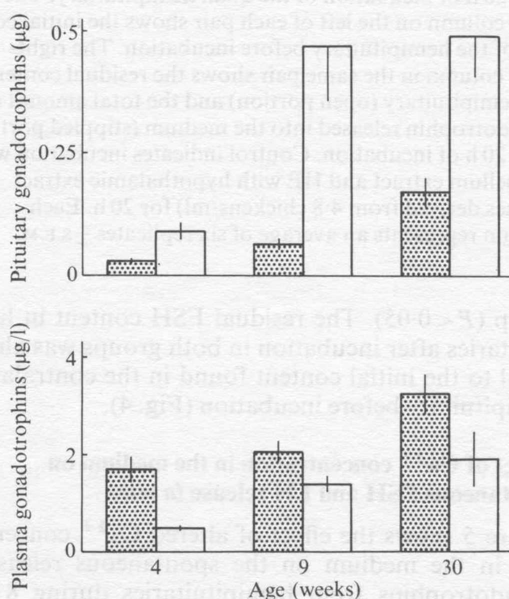

FIGURE 1. Contents of FSH (stippled bars) and LH (open bars) in the pituitary gland, and plasma concentrations of FSH and LH in male quail exposed to long days of 16L : 8D for different lengths of time from hatch. Each column represents an average of six birds ± S.E.M.

As shown in Fig. 2, the content of FSH in the pituitary glands from birds under short days was very low, only 2% of the amount of LH, but plasma FSH concentration was about twice that of LH. After photostimulation under 16L : 8D for 8 days, both pituitary content and plasma concentration of FSH and LH increased, but the relationship between content and concentration was always the same as that found before photostimulation.

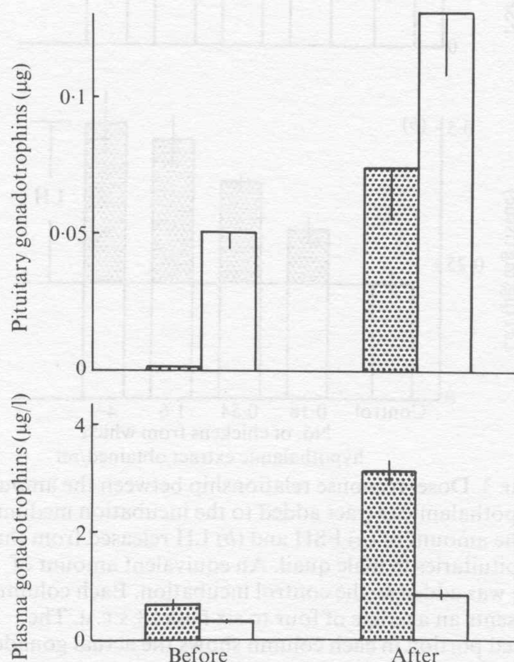

FIGURE 2. Changes in pituitary FSH (stippled bars) and LH (open bars) content and in plasma FSH and LH concentration in male quail after exposure to long days of 16L : 8D for 8 days (after). Before photostimulation, birds were killed on day 35 after being reared from hatch under short days of 8L : 16D (before). Each column represents an average of five birds ± S.E.M.

Effects of hypothalamic extract on FSH and LH release from quail pituitaries *in vitro*

In the first in-vitro experiment, amounts of gonadotrophins released into the media were estimated using different doses of hypothalamic extract (Fig. 3). The results show that the release of LH was clearly dependent on the dose of hypothalamic extract in the range employed (tissues derived from 0·18 to 4·9 chickens/ml), but the release of FSH was not clearly dose-dependent and the slope of the dose–response line was less steep than that for LH. The equation for the regression line of the dose–response relationship in the release of LH was $y = 139x + 426$ and that of FSH was $y = 21x + 174$. In this incubation system, 0·54 basal hypothalamic tissue/ml was the minimum dose which caused a significant ($P<0·01$ for LH, $P<0·05$ for FSH) increase in the release compared with the control.

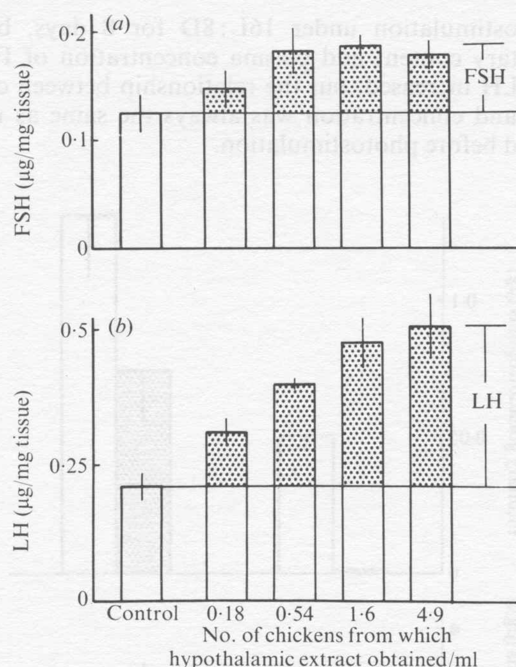

FIGURE 3. Dose–response relationship between the amount of hypothalamic extract added to the incubation medium and the amount of (*a*) FSH and (*b*) LH released from three hemipituitaries in male quail. An equivalent amount of saline was added to the control incubation. Each column represents an average of four to six flasks ± S.E.M. The stippled portion in each column shows the actual gonadotrophin release produced by the hypothalamic extract.

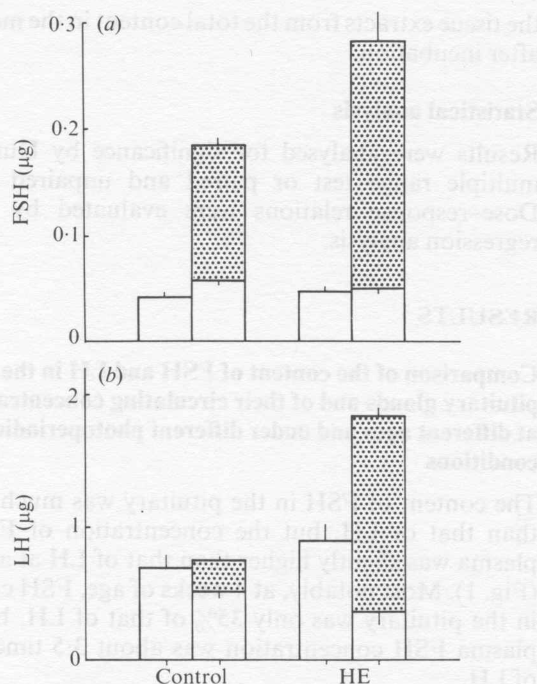

FIGURE 4. Release of (*a*) FSH and (*b*) LH and production after 20 h of incubation of the quail hemipituitary. The open column on the left of each pair shows the initial content of the hemipituitary before incubation. The right-hand column in the same pair shows the residual content of the hemipituitary (open portion) and the total amount of gonadotrophin released into the medium (stippled portion) after 20 h of incubation. Control indicates incubation with cerebellum extract and HE with hypothalamic extract (tissues derived from 4·8 chickens/ml) for 20 h. Each column represents an average of six replicates ± S.E.M.

Comparison of characteristics of release and production between FSH and LH from quail pituitaries *in vitro*

In the second experiment, incubation for 20 h was carried out to investigate differences in release and production between FSH and LH (Fig. 4). In the control group incubated with cerebellum extract, little LH was released into the culture medium during 20 h of incubation. In the presence of hypothalamic extract, the amount of LH released into the medium was six times higher than that of the controls ($P<0·001$). The residual LH content in hemipituitaries after incubation was decreased compared with the initial content in both groups, especially in the group treated with hypothalamic extract ($P<0·01$).

On the other hand, FSH was released into the culture medium even when hemipituitaries were incubated without hypothalamic extract (Fig. 4). In controls, the total amount of FSH released was more than three times the initial content of the hemipituitary ($P<0·001$). In the presence of hypothalamic extract, FSH released into the culture medium was only 1·8 times greater than the amount released in the control group ($P<0·05$). The residual FSH content in hemipituitaries after incubation in both groups was almost equal to the initial content found in the contralateral hemipituitary before incubation (Fig. 4).

Effect of Ca^{2+} concentration in the medium on spontaneous FSH and LH release *in vitro*

Figure 5 shows the effect of altered Ca^{2+} concentration in the medium on the spontaneous release of gonadotrophins from hemipituitaries during 8 h of incubation. Reducing the Ca^{2+} concentration from 1·8 to 0·3 mmol/l with EGTA significantly ($P<0·01$) decreased the spontaneous FSH release throughout the period of incubation. The effect of EGTA was cancelled by the addition of 1·5 mmol $CaCl_2$/l which restored Ca^{2+} concentration in the medium to 1·8 mmol/l. The mean LH release in the presence of EGTA became lower than that in the absence of EGTA after incubation for 6 to 8 h, and the difference was statistically significant ($P<0·05$).

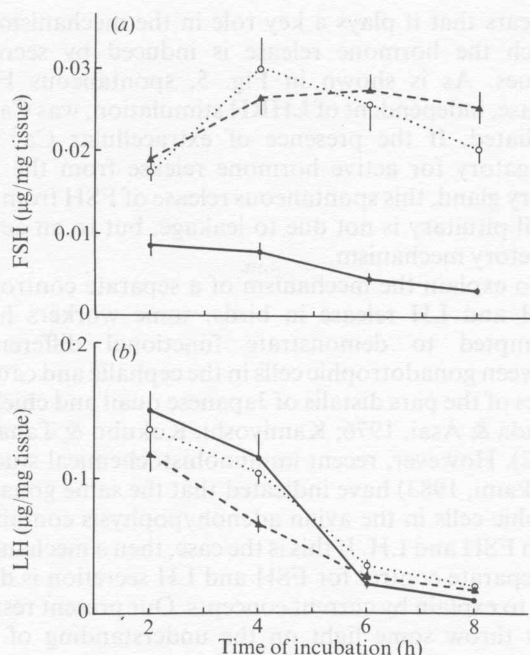

FIGURE 5. Effects of Ca^{2+} deprivation on the spontaneous release of (*a*) FSH and (*b*) LH from hemipituitary glands of quail incubated in medium containing 1·8 mmol Ca^{2+}/l (dotted lines), medium with the calcium ion reduced to 0·3 mmol/l by EGTA (solid lines), or medium with the calcium ion restored to 1·8 mmol/l by EGTA + $CaCl_2$ (broken lines) during 8 h of incubation. Each point represents an average of six flasks ± S.E.M.

DISCUSSION

The results show the different characteristics of FSH and LH in release and production. FSH appears to be spontaneously synthesized and released with minimal stimulation from the hypothalamus, whereas LH was stored in larger quantities and released under the influence of a hypothalamic-releasing hormone.

As shown in Figs 1 and 2, FSH was released in greater quantities into the general circulation than LH, especially in immature and non-photostimulated birds, even though the FSH content of the pituitary gland was much lower. In the present experiment, the amounts of LH and FSH are expressed in terms of one of the most highly purified chicken preparations so far obtained (LH: Hattori & Wakabayashi, 1979; FSH: Sakai & Ishii, 1980). The LH preparation, IRC-2 (Gunma), is considered to be immunologically as potent as the chicken LH preparation, IRC-2, of Follett, Scanes & Cunningham (1972) as reported by Goldsmith & Follett (1983). Our results show that a quantitative relationship between pituitary content and circulating concentration in quail are reversed for LH and FSH.

Gonadotrophin-releasing activity in the quail hypothalamus was first demonstrated by Follett (1970) using organ culture of chicken pituitaries. There have been several investigations since then which have confirmed that avian hypothalamic extracts stimulate LH release from superfused quail pituitaries (Smith & Follett, 1972), and from dispersed chicken pituitary cells (Bicknell & Follett, 1975). Bonney & Cunningham (1977*a*) used gentle mechanical dispersion of chicken anterior pituitary cells to study the mechanism of action of LH-releasing hormone (LHRH) in LH release. Following stimulation with mammalian LHRH, LH was released from the pituitary cells in a dose-related fashion. Cyclic AMP and dibutyryl cyclic AMP markedly enhanced the response of cells to mammalian LHRH, and antiphosphodiesterase compounds stimulated a release of LH whereas imidazole was inhibitory. These results suggest that the mechanism of action of mammalian LHRH in LH release from the chicken pituitary gland is similar to that suggested for mammals. Our present results also agree with these results; LH release was rather rigidly controlled by a gonadotrophin-releasing hormone in the hypothalamus (Figs 3 and 4). Chicken LH-releasing hormones have recently been isolated and shown to be molecules similar to mammalian LHRH (King & Millar, 1982; Miyamoto, Hasegawa, Minegishi *et al.* 1982 for chicken LHRH I; and Miyamoto, Hasegawa, Nomura *et al.* 1984 for chicken LHRH II). Chicken LHRH I and II, as well as mammalian LHRH, increase the release of LH from the pituitary gland of quail *in vitro* and *in vivo* (Ishii, Hattori, Wada *et al.* 1984; Hattori, Ishii, Wada *et al.* 1985).

There have been no reports on the mechanism of FSH release from the avian pituitary gland *in vitro*, since there are no appropriate sensitive radioimmunoassay systems except the one devised by Follett (1976). Sakai & Ishii (1983) recently established a chicken FSH RIA system, which has allowed us to study the mechanism of FSH secretion from the quail pituitary. The results obtained here indicated that FSH was released spontaneously (Fig. 4). This phenomenon was also suspected from the results obtained in mammals *in vivo* and *in vitro*. McCann, Cooper, Harms *et al.* (1974) reported that lesions in the rat median eminence region resulted in a decrease of approximately 95% in plasma LH *in vivo* but of only 75% in plasma FSH. In-vitro studies in men (Pasteels, Sheridan, Gaspar & Franchimont, 1977) and rats (Sheridan, Loras, Surardt *et al.* 1979) also suggested that FSH release was somewhat autonomous. Sheridan *et al.* (1979) employed long-term organ culture for 18 weeks to study FSH and LH release by pituitaries isolated from hypothalamic control in ovariectomized rats. They reported that in a medium favouring prolonged survival of the cultures,

FSH production initially decreased, but after 3 weeks this decrease progressively slowed down until the tenth week. It then began to increase and reached a plateau which persisted until the end of the culture period. The LH content of the medium fell to a low level within a few days in the same culture. More recently, Apfelbaum (1983) studied the time-course of release and synthesis of LH, FSH and prolactin for different incubation time-intervals of between 0 and 4 h in pituitary glands obtained from ovariectomized rats. He showed that prolactin-secreting cells had a very fast turnover rate and LH-secreting cells a low turnover rate; FSH-secreting cells had a rate intermediate between the two. In the present experiment, we used pituitaries from adult male quail which had been exposed to endogenous hypothalamic LHRH because the birds had been kept on long days before the glands were removed. It might be considered that the pituitaries in our in-vitro experiment were too sensitive to exogenous stimulation because of their previous exposure to endogenous LHRH. This possibility was excluded, however, by a preliminary experiment in which we also found that FSH release was spontaneous in the pituitary glands of immature male quail. These results in avian and mammalian species indicate that spontaneous FSH release is autonomous in the FSH-producing cells of the adenohypophysis. This does not mean, however, that FSH secretion is completely spontaneous; chicken hypothalamic extract enhanced FSH secretion from quail pituitary glands (Figs 3 and 4). We have found that a single injection of either chicken LHRH I or II into Japanese quail causes a rise in plasma FSH concentration as well as that of LH, suggesting that FSH secretion is also stimulated by endogenous hypothalamic LHRH (Ishii *et al.* 1984; Hattori *et al.* 1985). However, the in-vitro administration of hypothalamic extract (Figs 3 and 4) and the injection of chicken LHRH I or II into quail causes a much greater increase of LH than of FSH. This suggests that FSH secretion is controlled to a lesser extent by the hypothalamus than is LH. There was no significant difference in these activities between chicken LHRH I and II.

It has been shown repeatedly that removal of Ca^{2+} from the media leads to suppression of the release of pituitary hormones by various secretagogues (for review see Moriarty, 1978). Recently, de Koning, Tijssen, van Dieten & van Rees (1982) showed that the chelation of Ca^{2+} by the addition of EGTA resulted in a markedly depressed response of LH release from female rat pituitary glands in response to LHRH. Similar results were obtained with the chicken anterior pituitary gland stimulated by LHRH in Ca^{2+}-free (Bonney & Cunningham, 1977*b*) and Ca^{2+}-reduced (Luck & Scanes, 1980) medium. The exact role of Ca^{2+} in hormone release is still a matter of debate, but it appears that it plays a key role in the mechanism by which the hormone release is induced by secretagogues. As is shown in Fig. 5, spontaneous FSH release, independent of LHRH stimulation, was Ca^{2+}-mediated. If the presence of extracellular Ca^{2+} is obligatory for active hormone release from the pituitary gland, this spontaneous release of FSH from the quail pituitary is not due to leakage, but to an active secretory mechanism.

To explain the mechanism of a separate control of FSH and LH release in birds, some workers have attempted to demonstrate functional differences between gonadotrophic cells in the cephalic and caudal lobes of the pars distalis of Japanese quail and chicken (Wada & Asai, 1976; Kamiyoshi, Kokubo & Tanaka, 1982). However, recent immunohistochemical studies (Mikami, 1983) have indicated that the same gonadotrophic cells in the avian adenohypophysis contained both FSH and LH. If this is the case, then a mechanism of separate control for FSH and LH secretion is difficult to explain by current concepts. Our present results may throw some light on the understanding of the mechanism underlying the separate regulation of FSH and LH secretion and synthesis.

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