Changes in Specific Gonadotropin Binding Sites in the Liver during Metamorphosis in the Bullfrog, *Rana catesbeiana*

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**ABSTRACT**—The author studied bindings of radioiodinated bullfrog follicle-stimulating hormone (fFSH) and luteinizing hormone (fLH) to a crude plasma membrane fraction of the liver of tadpoles of various stages and adults of the bullfrog, *Rana catesbeiana*. Slight specific bindings of the labeled fFSH and fLH were detected in tadpoles between Stage V and XX. Specific bindings of the radioiodinated hormones increased significantly from Stage XXI, which is just after "climax" of metamorphosis. Furthermore, accumulation of 3'-5' cyclic adenosine monophosphate was stimulated by both fFSH and fLH in small blocks of the liver derived from individuals after the climax of metamorphosis but not in those derived from individuals before the climax. These results strongly suggest that, in bullfrogs after the climax of metamorphosis, gonadotropins act on the liver through specific membrane receptors coupled with the adenylate cyclase system, and stimulate some biochemical process in the liver.

**INTRODUCTION**

Challenging the general belief that distribution of gonadotropin receptors in the body of vertebrates is restricted to the gonads, Kubokawa and Ishii [1] demonstrated that in adult amphibians the liver as well as the gonad has specific receptors for gonadotropins, and also that bullfrog follicle-stimulating hormone (fFSH) and luteinizing hormone (fLH) can stimulate accumulation of 3',5'-cyclic adenosine monophosphate (cAMP) in the liver in adult bullfrogs. They showed that hormone-binding properties of these hepatic gonadotropin receptors are almost identical to those of gonadal gonadotropin receptors. Thus, they demonstrated that specific bindings of gonadotropins to the hepatic receptor are not nonspecific uptake of glycoproteins, and should play a specific physiological role, although it is unknown. Beside our study, extragonadal gonadotropin receptors was shown in the pig by Ziecik et al. [2]. They estimated that in pregnancy the total number of specific gonadotropin binding sites in the uterine tissue is equal to or higher than the total number of the sites in the ovaries. However, none of these studies has revealed the final physiological action of gonadotropin in these organs mediated by the extragonadal gonadotropin receptors.

Since amphibians show drastic metamorphosis, it is important and interesting to know the time of occurrence of the hepatic gonadotropin receptors through the life of the bullfrog. The present author studied change in gonadotropin bindings to the liver, if any, during the metamorphosis of the bullfrog.

**MATERIALS AND METHODS**

**Materials**

Bullfrog (*Rana catesbeiana*) tadpoles were purchased from a commercial dealer. They had been kept under a constant temperature (25°C) and light condition (12L:12D) for several days to several months until they reached appropriate stages, and then used for experiments which were performed between February and May, 1988. Stages of the tadpoles at the time of experiments were V, VI, and XIV-XXV of Taylor and Kollros...

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Adult male bullfrogs of over several months after metamorphosis were also purchased and used.

**Hormones and chemicals**

Bullfrog FSH and LH used were FF1341B and FL421B of Takada and Ishii [4], respectively. A gonadotropin rich glycoprotein fraction was prepared from bullfrog pituitaries according to Takada and Ishii [4] and used as competitor for obtaining nonspecific binding. Carrier-free Na\(^{125}\)I was purchased from Amersham Japan Corporation, Tokyo. An assay kit for radioimmunoassay of cAMP was purchased from Yamasa Shouyu Corporation, Chiba. As an inhibitor of phosphodiesterase, 1-methyl-3-isobutylxanthine (MIX) of Sigma Chemical Co. Ltd. was used.

**Iodination**

Bullfrog FSH and LH were radioiodinated by the lactoperoxidase (LPO) method [5]. Briefly, 2.5 \(\mu\)g of bullfrog FSH or LH was reacted with 0.5 \(\mu\)g LPO and 0.2 mCi of Na\(^{125}\)I for 1 min at room temperature. Free and bound iodide were separated by a 1.5 x 20 cm column of Sephadex G-75. Specific radioactivities of radioiodinated fFSH and fLH were estimated by matching data of competition and saturation experiments as described by Kubokawa and Ishii [5]. Specific radioactivities of \(^{125}\)I-fFSH and fLH were about 40 \(\mu\)Ci/\(\mu\)g.

**Plasma membrane preparation**

Tadpoles of different stages and adult males were anesthetized in ice water and decapitated. Livers were dissected out immediately, washed in chilled amphibian saline, and put into a plastic tube individually numbered. They were frozen by immersing in liquid nitrogen and stored at \(-80^\circ\)C until use.

Each frozen liver was thawed, weighed and homogenized for 1 min with micro homogenizer (Muromachi Co. Ltd., Tokyo) in 5 volumes of chilled 40 mM Tris-HCl buffer, pH 7.4 containing 50 mM \(\text{MgSO}_4\) (receptor buffer). The homogenate was centrifuged at 10,000 \(\times\) g for 20 min at 4°C. The pellet was washed by suspending in 20 ml of buffer and centrifuging again. The washed pellet was resuspended in the receptor buffer at the concentration of 10 mg of the original fresh tissue in 100 \(\mu\)l, and used as the crude plasma membrane preparation for binding experiments. The tissue concentration in the membrane preparation ranged between 10 mg and 20 mg per 100 \(\mu\)l among samples of different metamorphic stages.

**Binding experiments**

One hundred microliters of the plasma membrane preparation were mixed with 50 \(\mu\)l of \(^{125}\)I-fFSH or \(^{125}\)I-fLH (ca. 10,000 cpm) and 50 \(\mu\)l of the bullfrog pituitary glycoprotein solution containing 5 \(\mu\)g of the glycoprotein or the same volume of buffer alone. The radioligands and the pituitary glycoprotein were dissolved in 40 mM Tris-HCl, pH 7.4, containing 50 mM \(\text{MgSO}_4\) and 0.1 % BSA (incubation buffer). The mixture was incubated for 2.5 hr at 20°C when \(^{125}\)I-fLH was used, and for 17 hr at 15°C when \(^{125}\)I-fFSH was used. Membrane-bound hormone was separated from free hormone by centrifugation at 10,000 \(\times\) g for 3 min after addition of 1 ml of chilled incubation buffer. The pellet was washed twice in an aliquot of the buffer, and then counted for radioactivity. Specific binding was determined by subtracting nonspecific binding (binding of radioligand under the presence of the pituitary glycoprotein) from the total binding (binding of the radioligand under the absence of the pituitary glycoprotein). All the determinations were done in duplicate.

**Experiments on effects of fFSH and fLH on the cAMP concentration in the liver**

Livers were dissected out from tadpoles of Stage XVIII, XIX, XX, XXI, XXIV and adults (3–6 months old). Each fresh liver was minced finely into 1–2 mm cubes and washed several times by decanting and adding chilled amphibian Krebs-Ringer bicarbonate (aKRB). The tissue cubes were dispersed in aKRB as to 1 ml of the dispersion contains about 80 mg of the tissue. An aliquot of 250 \(\mu\)l of the cube dispersion was transferred to each well of a 24-well microplate. One hundred microliters of fLH or fFSH solution in aKRB containing 1 \(\mu\)g of hormone and 150 \(\mu\)l of aKRB containing 10 mM MIX were added to each well. Then, the microplates were incubated for 2 hr at 25°C with shaking under 95% \(\text{O}_2\) and 5% \(\text{CO}_2\).
After the incubation, liver blocks were collected from each well and used for determination of cAMP as follows. The liver blocks were homogenized in one milliliter of a 6% trichloro acetic acid solution, and cAMP in the tissue was extracted. The homogenate was centrifuged at 3,000 rpm for 5 min, and the resulting supernatant was transferred to a glass tube. Five volumes of water saturated with ethyl ether was added to each tube, and the tube was vigorously shaken for removing lipid. The aqueous layer was separated, and cAMP in the aqueous layer was determined by radioimmunoassay using the kit according to the maker's instruction. The protein concentration in the tissue blocks was determined by the Follin-Lowry method after solubilizing the tissue blocks in 2N NaOH. Bovine serum albumin was used as standard.

RESULTS

Change in the liver weight during metamorphosis

The liver weight varied among stages (the mean ranging between 0.15 and 0.35 g) (Fig. 1). It also varied widely among individual tadpoles in most stages. Accordingly the variation in the mean among stages was not statistically significant (P > 0.05).

Bindings of fFSH to liver membrane preparation of tadpoles at different stages

Specific binding of fFSH was extremely low until Stage XIX, although it was detectable throughout the metamorphic stages studied (Fig. 2). From Stage XXI, the specific binding started to increase and exceeded the nonspecific binding level from

![Graph](image-url)
Stage XXIV. The specific binding level at Stage XXV was about a half of that in the adult. Over-all difference in specific binding of fFSH to tadpole liver among metamorphic stages was statistically highly significant (P<0.01 by one-way layout analysis of variance after log-transformation of data). Kendall’s rank correlation coefficient (r) between metamorphic stages and mean specific bindings after Stage XIX was 0.905 and statistically highly significant (P<0.01). Nonspecific binding of fFSH also showed statistically significant variation among stages, but Kendall’s rank correlation coefficient (r=0.047) between stages and mean nonspecific bindings after Stage XIX was not statistically significant (P>0.05).

**Bindings of fLH to liver of tadpoles at different stages**

Changes in specific binding of fLH with stage were, as a whole, similar to those of fFSH. The specific binding was low in early stages, and started to increase from Stage XXI (Fig. 3). However, in fLH bindings, nonspecific levels were higher than those in fFSH bindings, and the rate of the change in the specific binding of fLH with stage was less marked than that of fFSH. By the final stage of metamorphosis, the specific binding of fLH had also reached a level about a half of the adult level. Variation of the specific binding of fLH among stages of tadpoles was statistically highly significant (P<0.01) when tested by the Kruscal-Wallis test. Kendall’s rank correlation coefficient (r=0.905) between metamorphic stages after Stage XIX and mean specific bindings was also highly significant (P<0.01). Variation of the nonspecific binding among stages of tadpoles was also statistically highly significant (P<0.01), when tested by the analysis of variance. However, Kendall’s rank correlation coefficient (r=0.52) between metamorphic stages and nonspecific bindings after Stage XIX was not significant (P>0.05).

**Effects of fLH and fFSH on the concentration of cAMP in liver**

Changes in the cAMP concentration in liver in response to gonadotropin treatments were expressed in terms of the ratio of the cAMP concentration in hormone treated tissue to that in corresponding untreated tissue (Figs. 4, 5). Data of Stages XVIII to XX were combined, since only a single determination was performed in Sage XVIII and XIX, and also gonadotropin bindings did not change so much until Stage XX. In individuals at Stage XVIII to XX, the cAMP concentration in the liver was not influenced by both fLH and fFSH treatments, the ratio to the control being about 1. In contrast, the cAMP concentration was increased by both of the treatments in the liver of individuals at and after Stage XXI. The difference in the response from the youngest Stage (Stage XVIII-XX) group was significant in Sage XXI and the adult stage in the FSH treatment (Fig. 4), and also in Sage XXI, XXIV and the adult stage in the LH treatment (Fig. 5). The concentration of cAMP in the untreated control tissues varied within a small range (the mean and standard deviation being 0.062 and 0.014 pmol per mg protein,
The liver of tadpoles and adults of the bullfrog. Changes in the cAMP concentration are expressed in terms of the mean of ratios to control. The number of repetitions is indicated in parentheses under the stage number. The vertical bar at the top of each column shows the standard error of the mean.

* Difference from the youngest stage group is significant at $P<0.05$.

** Difference from the youngest stage group is significant at $P<0.01$.

Fig. 4. Effect of bullfrog FSH in vitro on the cyclic AMP concentration in the liver of tadpoles of different stages and adults of the bullfrog. The cAMP concentration is expressed in terms of the mean of ratios to control. The number of repetitions is indicated in parentheses under the stage number. The vertical bar at the top of each column shows the standard error of the mean.

* Difference from the youngest stage group is significant at $P<0.05$.

** Difference from the youngest stage group is significant at $P<0.01$.

Fig. 5. Effect of bullfrog LH in vitro on the cyclic AMP concentration in the liver of tadpoles of different stages and adults of the bullfrog. Changes in the cAMP concentration are expressed in terms of the mean of ratios to control. The number of repetitions is indicated in parentheses under the stage number. The vertical bar at the top of each column shows the standard error of the mean.

* Difference from the youngest stage group is significant at $P<0.05$.

** Difference from the youngest stage group is significant at $P<0.01$.

DISCUSSION

In the present study, significant amounts of specific gonadotropin bindings were demonstrated in the liver of bullfrog larvae of the late stages after Stage XXI. Slight specific bindings of FSH and LH to the liver were observed in larvae of the younger stages before Stage XXI. However, the specific bindings to the liver of the younger stage larvae are too small and hence considered to be physiologically insignificant. Similar low levels of specific bindings of gonadotropins have been detected in a number of non-gonadal and non-hepatic tissues in various vertebrates (in rat [6], turtle [7], rainbow trout [8] and bullfrog [9]). The increase in the specific bindings of FSH and LH at Stage XXI is considered to indicate appearance of specific gonadotropin receptors in the hepatic tissue. This conclusion is supported also by the cAMP experiment. The hepatic tissue started to respond to gonadotropins by increasing the cAMP concentration from Stage XXI.

Stage XX, the stage just before gonadotropin receptors appear in the liver, has been referred to the onset of metamorphic climax. A number of physiological changes occur in the bullfrog larva around this stage. For example, Kistler et al. [10] reported that a steep increase in the free amino acid concentration took place in the liver around this stage. An increase in secretion of glucocorticoids toward the climax was reported by Jaffe [11]. Yamamoto and Kikuyama [12] revealed that the concentration of prolactin in plasma increased rapidly from Stage XXII and reached the maximum at Stage XXIV. However, it is difficult to correlate a certain one among these phenomena to the appearance of gonadotropin receptors in the liver.

Janssens and Maher [13] found in the adult axolotl that glucagon and adrenaline increased the cAMP concentration in the liver. Janssens and Grigg [14] reported in the adult *Xenopus* that adrenaline stimulated glycogenolysis in the liver in vitro. The results by the present author in conjunction with these previous results suggest a possibility that gonadotropin mimes or synergistically
intensify the effect of glucagon or adrenalin in the liver in bullfrogs after the metamorphic climax. This possibility should be tested by examining in vitro effects of frog gonadotropins and cyclic nucleotide on the carbohydrate metabolism in liver of bullfrogs after the climax of the metamorphosis.

It will be interesting to compare the change in the hepatic gonadotropin receptors with changes in hepatic receptors for the other pituitary hormones. White and Nicoll [15] failed to detect significant specific bindings of prolactin in the liver of bullfrog tadpoles at any stage, while they demonstrated high specific bindings in other organs such as tail, gill and kidney. Carr et al. [16] detected specific bindings of prolactin in the liver as well as in the tail and kidney. However, the level of the binding was lowest in the liver. They further showed that the number of prolactin binding sites in the tadpole liver increased during early metamorphic stages and decreased at the climax of metamorphosis. No study has been reported for receptors for the other pituitary hormones in the tadpole liver.

As far as hepatic gonadotropin receptors appear at Stage XXI and if these receptors play some physiological role, synthesis and secretion of gonadotropins should have started before this stage. An immunocytochemical study showed that FSH and LH became detectable from Stage V and X, respectively [17]. This study also revealed with radioimmunoassay that the content of FSH had a peak at Stage XVIII and that of LH at around Stage XXIII. Thus, synthesis and secretion of gonadotropins precedes the appearance of hepatic gonadotropin receptors. Unfortunately, change in plasma gonadotropin levels in bullfrog tadpoles is not clear. Plasma concentrations of gonadotropins in bullfrog tadpoles of Stage XXIII to XXV were low, ranging between 0.3 and 0.8 ng/ml in LH and between 1.0 and 2.2 ng/ml in FSH, and it was difficult to detect the change clearly (personal communications with Dr. S. Tanaka, Gunma University, Maebashi). Furthermore, no ontogenic study of gonadal gonadotropin receptors was reported in amphibians, although sex differentiation of the gonads has been reported to start in the late metamorphic stage in Rana nigromaculata [18].

In conclusion, the hepatic gonadotropin receptors of the late metamorphic stage larvae of the bullfrog seem to have some physiological function as well as those of the adult bullfrog. Further biochemical study to find the final hepatic function that is mediated by the gonadotropin receptors is needed.

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REFERENCES


Gonadotropin Receptors in Tadpole Liver

Biochem., 12: 395-400.


