A Homologous Radioimmunoassay for Bullfrog Basic Gonadotropin

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Abstract

A double antibody radioimmunoassay (RIA) for the measurement of bullfrog basic gonadotropin (GTH) is described. The antiserum was raised against a highly purified bullfrog basic GTH. Bullfrog basic GTH-IV ($pI=9.33$) was used for radioiodination and as a reference standard. The dissociation constant of the binding of the antibody to bullfrog basic GTH-IV was $8.28 \times 10^{-12} M$. The standard curve was obtained with 0.1–25.6 ng/ml of bullfrog basic GTH-IV. When bullfrog sera were assayed by this RIA system, the intra-assay coefficient of variation and inter-assay coefficient of variation were 4.45% and 5.6%, respectively. The accuracy of the assay was shown as the recovery of bullfrog basic GTH-IV added to the pooled bullfrog sera. With a range of 0.1–12.8 ng/ml, the recovery was $104.78 \pm 11.91\%$ (Mean $\pm$ S.D.). When bullfrog pituitary extract was fractionated by isoelectric focusing (IEF) and assayed for gonadotropin by this RIA system and also by radioreceptor assay systems employing *Xenopus* or *Anolis* testicular homogenates, the RIA positive peaks agreed well with those obtained with *Xenopus* RRA, indicating that the present RIA system measures LH-like gonadotropin. The inhibition curves of pituitary preparations from bullfrog, brown frogs and dark-colored frog were almost parallel to the standard curve, while those of other anuran and urodele species were not. This RIA system was highly sensitive, but allowed only a narrow application, because of species-specificity.

Bioassays for amphibian pituitary gonadotropins are very often insufficient in their sensitivity and precision for the measurement of circulating gonadotropins. If a highly sensitive radioimmunoassay (RIA) system is developed for amphibian gonadotropin, it must be very useful for endocrinological studies. Because of species-specificity, in the development of an RIA system for amphibian gonadotropins, it was necessary to obtain a highly purified gonado-

tropins from pituitaries of amphibians. Licht and his co-workers have purified gonadotropins from pituitaries of three species of amphibians (Licht and Papkoff, 1974; Licht *et al.*, 1975; Papkoff *et al.*, 1976; Farmer *et al.*, 1977). Daniels *et al.* (1977; 1980) reported first homologous RIA systems for LH and FSH of *Rana catesbeiana*, however, their assay performance, and affinity and species-specificity of the antisera were not described, and those assay systems were not available to us. Recently, Takahashi and Hanaoka (1981) obtained a highly purified bullfrog basic gonadotropin (GTH) from pituitaries of *R. catesbeiana*. In the present

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study, we developed a sensitive RIA system for bullfrog basic GTH and examined the crossreactivity of pituitary preparations from various species of amphibian to this RIA system.

Materials and Methods

Preparation of bullfrog basic gonadotropin (GTH)

The previous report (Takahashi and Hanaoka, 1981) revealed that bullfrog basic GTH is composed of four components with pIs of 8.80, 8.96, 9.11 and 9.33, which are tentatively termed bullfrog basic GTH-I, II, III and IV, respectively. In the *Xenopus* ovulation bioassay and in the *Xenopus* radioreceptor assay employing ^{125}I -rat FSH as radioligand and *Xenopus* testicular homogenates as receptor preparation, basic GTH-I, II and III had similar potencies to that of NIH-LH-B10, while basic GTH-IV was 2–3 times as potent as the other three components (Takahashi and Hanaoka, 1981). In this study, the basic GTH-IV was used as the hormone for radioiodination and as a reference standard.

Production of the antiserum

A highly purified bullfrog basic GTH-M, which is a mixture of basic GTH-I, II, III and IV preparation, was used to raise antisera in three rabbits. Two hundred micrograms of the hormone, dissolved in 0.5 ml of saline, was emulsified in 0.5 ml of Freund's complete adjuvant with 0.25 mg *M. Butyricum* (DIFCO, Detroit, Mich.) and was given to a rabbit in the several intradermal sites of the back weekly for 2 months. Blood samples were obtained from the ear vein before each injection and tested for antibody titer. After 2 months, the amount of GTH-M was increased to 400 μg and was injected six times at 10-day intervals. Eight days after the final injection, the rabbits were bled from the carotid arteries. The sera were separated by centrifugation, added to 0.01 % merthiolate and stored at -70°C .

Radioiodination

Radioiodination of the purified bullfrog basic GTH-IV were carried out according to the chloramine T method of Greenwood *et al.* (1963) with a minor modification. The labeled hormone was separated with Biogel P-60 (Bio. Rad. Lab., Richmond, Calif.) column (Disposable plastic column; Whale Sci. Inc.).

Radioimmunoassay system

A double antibody radioimmunoassay system was established. The first incubation was carried out at room temperature overnight after mixing 400 μl of 1 % BSA (bovine serum albumin) in phosphate

buffered saline (PBS: 0.01 M sodium phosphate and 0.14 M NaCl containing 0.01% merthiolate, pH 7.5), 200 μl of the standard hormone dissolved in 1 % BSA-PBS or assay samples, 100 μl of the first antiserum diluted with 1% NRS (normal rabbit serum) in 0.05 M EDTA-PBS and 100 μl of radioiodinated hormone dissolved in 1% BSA-PBS. After the first incubation, 200 μl of goat anti-rabbit gamma-globulin serum (prepared in the Hormone Assay Center) diluted with 0.05 M EDTA-PBS was added, mixed and incubated at 4°C overnight. The assay tubes were then centrifuged at 3,000 rpm for 15 min and the supernatant fluid was removed by suction. The precipitate was counted for radioactivity in an automatic gamma counter (Beckmann 8000). The assay results were expressed as nanograms of bullfrog basic GTH-IV.

Radioreceptor assay (RRA)

For the measurement of GTH activity, the two RRA systems described in the previous report were used (Tanaka *et al.*, 1981); one with *Xenopus laevis* testicular homogenate as the receptor preparation which is thought to be specific to LH-like GTH (Adachi *et al.*, 1979), and the other with *Anolis carolinensis* testicular homogenate which seems to be specific to FSH-like GTH, both employing ^{125}I -labeled rat FSH as radioligand.

^{125}I -labeled rat FSH was prepared by radioiodination of NIADDK rat I-3 (supplied by the National Pituitary Agency and the Pituitary Hormones and Antisera Center, NIADDK, NIH) with lactoperoxidase according to the method of Miyachi *et al.* (1972) with minor modification. Nonspecific binding was determined by using 100 I.U. of pregnant mare serum gonadotropin (Sankyo Manufacturing Co.). Results were expressed as micrograms of NIADDK rat FSH I-3.

Isoelectric focusing (IEF) fractionation

Pituitary extract was prepared by homogenizing one gland from an adult male bullfrog collected early in April with 1 ml of water. The insoluble fragment was removed by centrifugation at 15,000 rpm for 20 min. The IEF fractionation was carried out on 25 ml column with Ampholine pH 3.5–10 (LKB Produkter) at a 1 % concentration, according to the method described in the previous report (Tanaka *et al.*, 1981). After focusing, the solution was eluted at a flow rate of 0.1 ml/min, and 0.45 ml fractions were collected. At the same time, the pH of each fraction was automatically measured. These fractions were diluted 1:10 with Tris-MgCl₂-BSA [40 mM tris-(hydroxymethyl)-aminomethane HCl, 5 mM MgCl₂, pH 7.5, containing 1% BSA] and stored at -20°C until assay for gonadotropins. These stored sample solutions were further diluted to appropriate volumes with Tris-MgCl₂-BSA or 1 % BSA-PBS prior to assay.

Fig. 1. A standard curve with bullfrog basic GTH-IV and an inhibition curve obtained with sera from bullfrogs. Each point of standard curve represents mean $\pm$ S.D. of triplicated tubes.

Hormone preparations

To elucidate the specificity of the RIA for bullfrog basic GTH, NIADDK rat FSH I-3, NIADDK ovine LH #21 and bullfrog acidic GTH ($pI=6.2$, prepared by Takahashi and Hanaoka, 1981) were used for competitive inhibition of the binding of labeled bullfrog basic GTH-IV.

Pituitary extracts

The pituitary glands were obtained from amphibians including anuran species; *Rana catesbeiana*, brown frogs (*Rana ornativentris*, *Rana japonica*), dark-colored frog (*Rana rugosa*), pond frogs (*Rana nigromaculata*, *Rana breviposa*, *Rana brevipoda porosa*), other anuran species (*Bufo bufo japonicus*, *Hyla arborea japonica*, *Bombina orientalis*, *Xenopus laevis*), and urodele species (*Cynops pyrrhogaster pyrrhogaster*, *Aneides areneus*). The glands were homogenized with PBS, then frozen and thawed, and centrifuged at 15,000 rpm for 15 min. The supernatant fluid was stored at -20°C until use. Prior to assay, a part of the supernatant fluid was serially diluted with 1% BSA-PBS.

Results

When the antiserum diluted at 1 : 128,000 was incubated with labeled hormone (c. a. 30,000 cpm/tube) in this assay system, approximately 30% of labeled hormone was bound. Therefore, in the following assay, this dilution of antiserum was used. Fig. 1 shows a standard curve obtained with 0.1–25.6 ng/ml of bullfrog basic GTH-IV. The sera obtained from adult bullfrogs were assayed by this system in triplicates in three different dilutions. The inhibition curves obtained with these samples were fairly parallel to the standard curve obtained with bullfrog basic GTH-IV. With a range of 0.1–12.8 ng/ml, the recovery of bullfrog basic GTH-IV added to the pooled bullfrog sera was $104.78 \pm 11.91\%$ (mean $\pm$ standard deviation) (table 1). The mean within-assay

Table 1. Recovery Test

	Number of tubes	Hormone added (ng/ml)	Assay value (ng/ml)	Hormone found (ng/ml)	Recovery (%)
Serum alone	3	0	0.495	—	—
Serum + Hormone	3	0.1	0.622	0.127	127
"	3	0.2	0.680	0.185	92.5
"	3	0.4	0.864	0.369	92.3
"	3	0.8	1.258	0.763	95.4
"	3	1.6	2.095	1.062	100.0
"	3	3.2	3.802	3.307	103.3
"	3	6.4	7.439	6.944	108.5
"	3	12.8	15.757	15.264	119.2

Accuracy of the RIA is shown as the recovery of bullfrog basic GTH-IV added to the pooled bullfrog sera. Within a range of 0.1–12.8 ng/ml, the recovery percentage was $104.78 \pm 11.91\%$ (Mean $\pm$ S.D.).

Table 2. Precision and Reproducibility

Bullfrog serum (1)

Trial	N	ng/ml	C. V.
1st.	3	6.631	0.28
2nd.	3	5.921	6.57
3rd.	3	7.105	19.79
4th.	3	6.167	4.39
C. V.		8.1%	

Bullfrog serum (2)

Trial	N	ng/ml	C. V.
1st.	3	16.947	0.52
2nd.	3	16.868	1.66
3rd.	3	17.859	0.35
4th.	3	16.637	2.04
C. V.		3.1%	

Then mean within-assay coefficient of variation determined from eight triplicates of bullfrog serum samples was 4.45%. The mean between-assay coefficient of variation estimated from the results of 4 consecutive assays of 2 serum samples was 5.6%.

coefficient of variation determined from eight triplicates of bullfrog serum samples was 4.45%. The mean between-assay coefficient of variation estimated from the results of 4 consecutive assays of 2 serum samples was 5.6% (table 2).

Affinity of the antiserum to bullfrog basic GTV-IV

For the calculation of a specific activity of ^{125}I -labeled bullfrog basic GTH-IV, an aliquot of ^{125}I -labeled bullfrog basic GTH-IV was assayed by the RIA system with ^{131}I -labeled bullfrog basic GTH-IV as the labeled antigen. As a result, the labeled bullfrog basic GTH-IV had a specific activity of $140.2 \mu\text{Ci}/\mu\text{g}$. Fig. 2 shows a Scatchard plot drawn from a standard curve

obtained with bullfrog basic GTH-IV. The affinity constant (K_a) of the antiserum calculated from this Scatchard plot was $1.28 \times 10^{11} \text{ M}^{-1}$, and the dissociation constant (K_d) was $8.28 \times 10^{-12} \text{ M}$.

Crossreactivity of pituitary preparations with RIA system

Fig. 3 shows the effect of NIADDK rat FSH I-3, NIADDK ovine LH #21, bullfrog basic GTH-I, II, III and bullfrog acidic GTH. NIADDK rat FSH I-3 and NIADDK ovine LH #21 hardly affected the binding even when large amounts of the hormones were used. The crossreaction of bullfrog acidic GTH was about 10%. Inhibition curves of bullfrog basic GTH-I, II and III were fairly parallel to the standard curve obtained with

bullfrog basic GTH-IV.

IEF pattern of bullfrog GTHs (Fig. 4)

In the IEF pattern of GTH obtained by *Xenopus* RRA, two peaks of GTH, one

distinct peak and several minor peaks, were recognized at pH around 10.65–9.10 and 8.27–6.28, respectively. On the other hand, in the IEF pattern of GTH obtained by *Anolis* RRA, seven peaks of GTH, three distinct peaks and four less distinct peaks, were found in neutral to acidic pH region. The major components were at pH 7.06, 6.28 and 5.75. The minors were at pH 8.27, 5.43, 5.10 and 4.85. When the same IEF fractions were assayed by the present RIA system, we recognized 1 distinct peak at pH around 10.65–9.10 and 4 minor peaks at pH 8.27, 7.60, 6.94 and 6.34.

Fig. 2. Scatchard plot of the binding of bullfrog basic GTH-IV to the antiserum.

Crossreactivity of amphibian pituitary preparations with RIA system

In order to estimate the crossreactivity of gonadotropin of various amphibian species, the crude pituitary extracts were added to this assay system at various concentrations. Pituitary extracts of *Rana catesbeiana*, *R. ornativetris*, *R. japonica* and *R. rugosa* tested showed inhibition curves parallel with the standard curve obtained with bullfrog basic GTH-IV, while those of *R. nigromaculate*, *R. brevipoda* and *R. brevipoda porosa* did not

Fig. 3. Competitive inhibition curves of various gonadotropin preparations in the RIA for bullfrog basic GTH. Each point on curves represents mean $\pm$ S.D. of triplicated tubes.

Fig. 4. Isoelectric focusing patterns of pituitary gonadotropins in the adult male bullfrog. Gonadotropins were measured by the RIA (○—○) or *Anolis* (●—●) and *Xenopus* (●—●) RRA. Dots in the figure represent pH of the fractions.

(Fig 5). Furthermore, no reasonable crossreaction was observed with the extracts from *Bufo bufo japonicus*, *Xenopus laevis*, *Bombina orientalis*, *Hyla arborea japonica*, *Aneides aeneus* or *Cynops pyrrhogaster pyrrhogaster*.

Discussion

In amphibian, pituitary control of gonadal function was well established (Lofts, 1974). However, the specificity and action of gonadotropin regulating gonadal function is still not clear. One of the reasons may be that the assay methods for GTHs in plasma have been not sufficiently generalized. Although Daniels *et al.* (1977; 1980) established a homologous RIA for frog LH and FSH, the assay variation and application of the assay system was not reported in detail. Therefore, it was necessary to establish a sensitive RIA for amphibian gonadotropin in order to clarify plasma GTH levels. Recently, basic gonadotropin was highly purified from pituitaries of *Rana catesbeiana* (Takahashi and Hanaoka, 1981). Using the preparation, we developed a sensitive RIA system for

Fig. 5. Competitive inhibition curves of the pituitary extracts from *Rana* group in the RIA for bullfrog basic GTH. Samples were assayed in triplicate.

bullfrog basic GTH. Bullfrog basic GTH-M which was used to raise antiserum had the potency of mammalian LH-like hormone (Takahashi and Hanaoka, 1981). The binding of ^{125}I -labeled basic GTH to antiserum was not significantly influenced by highly purified ovine LH and rat FSH. The inhibition curves of bullfrog basic GTH-I, II and III were fairly paralleled to the standard curve obtained with bullfrog basic GTH-IV. The crossreaction with bullfrog acidic GTH was about 10%. This crossreaction probably is not due to contamination, since both bullfrog GTHs were highly purified. TSH contamination in bullfrog basic GTH-M used as immunogen was not clarified. It was reported that the bullfrog basic GTH-IV used as the hormone for radioiodination has a pI of 9.33 (Takahashi and Hanaoka, 1981). Generally, it was thought that TSH is not found in this high pH region. Therefore, the contamination of TSH must be negligible in the bullfrog basic GTH-IV preparation. Even if the anti-bullfrog basic GTH-M contained anti-bullfrog TSH, our RIA system should be fairly specific for bullfrog basic GTH owing to the use of bullfrog basic GTH-IV as the radioiodinated hormone. The IEF profile of bullfrog pituitary extract obtained with RIA also supported this view. When bullfrog GTH activity was assayed by the two RRA systems after IEF fractionation, *Xenopus* RRA-positive peaks were recognized in the alkaline region, while *Anolis* RRA-positive peaks were found in the neutral to acidic pH region. When the same IEF fractions were assayed by this RIA system, the positive peaks agreed well with those obtained with *Xenopus* RRA. As the *Xenopus* RRA system is thought to be specific to LH-like GTH in amphibians (Adachi *et al.*, 1979), the present RIA system measures LH-like GTH. In the present study, we used a wide pH range for IEF to show the IEF profiles of both basic and acidic GTHs. This made the separation of basic GTH I-IV insufficient because these basic GTHs

appeared in the most alkaline region where the pH resolution was poor. Precision of the assay is excellent with an inter-assay variation of 4.45%, the intra-assay coefficient of variation being 5.6%. The accuracy of the assay was shown as the recovery of bullfrog basic GTH-IV added to the pooled sera. With a range of 0.1–12.8 ng/ml, the recovery of $104.78 \pm 11.91\%$ (Mean $\pm$ S.D.) was sufficient. We examined the crossreaction of pituitary extract and serum from thirteen species in seven genera of amphibian. In *Rana catesbeiana*, the competition curves with pituitary extract and serum were parallel to that of standard bullfrog basic GTH-IV, indicating that this RIA system is able to measure basic GTH in plasma and pituitary from *Rana catesbeiana*. Furthermore, pituitary extracts from brown frogs (*R. japonica*, *R. ornativentris*) and dark-colored frog (*R. rugosa*) were almost parallel to the standard, but those of other anuran and urodele species were not. Therefore, this RIA system shows a high species-specificity, and does not allow wide application to measure the basic GTH of various species of amphibians. On the other hand, of special interest in the phylogeny of amphibians is the fact that pituitary extracts of the pond frogs i.e. *R. nigromaculata*, *R. brevipoda* and *R. brevipoda porosa* were not parallel to standard curve, because these species belong to the *Rana* group similar to bullfrog.

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