Radioimmunoassay of Prolactin in Plasma of Bullfrog Tadpoles

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Abstract

Antiserum to prolactin (PRL) of bullfrog (f), Rana catesbeiana, was produced by immunizing rabbits with the highly purified fPRL obtained from adenohypophyses of adult bullfrogs. In Ouchterlony's agar double diffusion test, a single precipitin line was produced between the fPRL antiserum and fPRL or the bullfrog pituitary extract. No precipitin line was produced between the antiserum and ovine PRL, bovine PRL, ovine growth hormone (GH) or fGH. The acetone-dried powder of bullfrog pituitary glands which had been incubated with the fPRL antiserum had much less potency in promoting collagen synthesis of the tail fin of bullfrog tadpoles than that incubated with normal rabbit serum. Histological studies on bullfrog adenohypophyses revealed that the cells that immunologically reacted with the antiserum to fPRL were erythrosinophilic.

The antiserum to fPRL was used to develop a radioimmunoassay (RIA) in which fPRL and 125I-fPRL were employed as the standard and the radioligand, respectively. Several dilutions of plasma of both adult and larval bullfrogs yielded dose-response curves which were parallel to the standard curve. Bovine PRL, ovine PRL, ovine GH and fGH did not react in this assay. Plasma from hypophysectomized bullfrogs had no detectable immunoreactive prolactin. Pituitary homogenates of Rana catesbeiana and Bufo bufo japonicus gave inhibition curves which were parallel to the standard. Pituitary homogenates of Xenopus laevis and Hynobius tokyoensis gave inhibition curves which did not parallel the standard. RIA of plasma PRL in larval bullfrogs of various developmental stages were performed. Average concentrations of prolactin during premetamorphosis (st. X), prometamorphosis (st. XVI-XIX) and at early climax stage (st. XX-XXII) were 18, 21-25 and 27-35 ng/ml, respectively. At advanced climax stage PRL levels were quite high. Average values at stages XXIII, XXIV and XXV were 98, 169 and 116 ng/ml, respectively. The significance of PRL levels in relation to metamorphosis is discussed.
fPRL and the RIA was applied to measure the plasma PRL levels in individual larval bullfrogs at various stages of development. In addition, cross reactivity of the pituitary homogenate of several amphibian species to the fPRL antiserum was studied.

Materials and Methods

Bullfrog prolactin

For immunization, iodination and reference preparation, prolactin was purified from bullfrog (Rana catesbeiana) adenohypophyses by extraction of acetone-dried powder with acid acetone and chromatography on DEAE-cellulose and Sephadex G100 as described previously (Yamamoto and Kikuyama, 1981).

Antibody production

Antiserum to fPRL were produced by immunizing four female white rabbits with highly purified fPRL. Each rabbit received 1 mg fPRL dissolved in 1.5 ml of saline and emulsified in an equal volume of Freund’s complete adjuvant (Difco). Injections were performed subcutaneously at multiple sites 3 times at 14 day intervals. Two weeks after the last injection, all animals were bled. The resulting antisera were tested against fPRL and other hormones by Ouchterlony’s immunodiffusion method (Ouchterlony, 1948). The antisera were also tested at various concentrations for their ability to bind 125I-fPRL and the antiserum with the highest titre was used in the experiments on inactivation of bioactivity of fPRL, immunochemical staining and RIA.

Immunodiffusion test

Immunodiffusion plate was made of 1% agar in 0.02 M phosphate buffer (pH 7.2). In the center well, 10 µl fPRL antiserum was placed. Each 10 µg of ovine(o) growth hormone(GH) (NIH-GH-S11), bovine (b)PRL(NIH-P-B6), oPRL(NIAMD-o-PRL-14), fGH and fPRL dissolved in 0.05 M bicarbonate-carbonate buffer (pH 10) was placed in the peripheral well. Adenohypophyses from three adult bullfrogs were homogenized in 0.3 ml of 0.05 M bicarbonate-carbonate buffer (pH 10) and centrifuged at 3,000 rpm for 10 min. Ten µl of the supernatant fraction was also placed in the peripheral well. The immunodiffusion plate was kept at 37°C overnight. Bullfrog GH used here is a fraction obtained during the purification of fPRL (Yamamoto and Kikuyama, 1981). It has a potent growth-promoting effect on Bufo and Xenopus juveniles.

Inactivation of bioactivity of fPRL by antiserum

Four hundred µg of acetone-dried bullfrog anterior pituitary powder dissolved in 100 µl of 1% BSA-PBS (bovine serum albumin-phosphate buffered saline, pH 7.5) or 100 µl of BSA-PBS was mixed with 100 µl of fPRL antiserum. An equal amount of pituitary powder in 100 µl of BSA-PBS or 100 µl of BSA-PBS was mixed with an equal volume of normal rabbit serum. These mixtures were incubated for 24 hr at 4°C. Premature morphic tadpoles (st. X) received three injections of either mixture every other day. Twenty four hr after the last injection, the tail fin was dissected out. Incorporation of 14C-proline into the collagen fraction in the tail fin in vitro was determined according to the method of Yoshizato and Yasumasu (1970).

Immunoochemical staining of PRL cells

Immunoochemical staining was carried out on the pituitary gland of bullfrogs fixed in Bouin-Holland sublimate according to the indirect peroxidase-labeled antibody method (Nakane and Pierce, 1967) using the fPRL antiserum and goat anti-rabbit globulin coupled to horseradish peroxidase (Cappel Laboratories). Of the mid-sagittal sections cut into 4 µm thicknesses, several sections were selected for immunoochemical staining and neighboring sections were stained with Cleveland and Wolfe’s trichrome.

Radioiodination of fPRL

The radioiodination of bullfrog prolactin was carried out at 20°C according to the method of Sakai et al. (1975). Two hundred µCi of Na125I (carrier free; The Radiochemical Centre Amersham, England) in 10 µl of 0.1 N NaOH was added to a polystyrene tube (10×70 mm) containing 25 µl of 0.5 M phosphate buffer (pH 6.9), 5 µg fPRL in 10 µl of 0.05 M phosphate buffer (pH 7.3) and 5 µg lactoperoxidase (Boehringer Mannheim) in 5 µl of 0.05 M phosphate buffer (pH 7.3). The reaction was started by adding 10 µl of 0.00025% hydrogen peroxide. To maintain the reaction, 10 µl of H2O2 was added 3 times at 1, 3 and 6 min. After 10 min of reaction, the iodination mixture was layered on a 12 ml column of Sephadex G75 (Pharmacia, Sweden) previously washed with 1 ml of 2% bovine serum albumin in 0.05 M phosphate buffer (pH 7.3) and equilibrated with the phosphate buffer. One ml aliquots were collected in tubes containing 0.2 ml of 2% bovine serum albumin. Specific activity of the radioiodinated fPRL was calculated by the method of Greenwood et al. (1963). Specific radioactivity of the labeled fPRL was about 40 µCi/µg.

Radioimmunoassay

For measuring fPRL in pituitary homogenates and plasma samples, the antiserum was used at a final dilution of 1 : 50,000. At this dilution the antiserum bound about 35% of the radioiodinated hormone in an assay tube containing no unlabeled hormone. The RIA was carried out in a disposable 10×70-mm polystyrene tube. Two hundred µl of diluent (1% BSA-PBS, pH 7.5) containing 0.5% normal rabbit serum and various amounts of hormones, pituitary homogenates or plasma samples in 100 µl of diluent were added to the assay
tubes. One hundred μl of a 1 : 10,000 dilution of antiserum to fPRL and approximately 0.2 ng of radioiodinated fPRL (10,000 cpm) in 100 μl of diluent were also added to each incubation tube. All assay tubes were incubated for 48 hr at 4°C. After incubation, 200 μl of a 1 : 100 dilution of goat antiserum to rabbit γ-globulin, which would maximally precipitate the antibody-bound radioiodinated hormone, was added and incubated for 24 hr at 4°C. After the second antibody reaction was completed, the assay tubes were centrifuged at 4°C for 30 min at 4,000 rpm and each supernatant was decanted. The precipitate was counted in an Aloka Auto Well Gamma System. The number of cpm in the tubes containing the antiserum and the labeled prolactin but no unlabeled prolactin was arbitrarily designated as 100% and the cpm in all tubes were expressed as a percentage of this number. Statistics for linearity, parallelism, precision and potency estimation in the parallel line assay were computed according to the method of Bliss (1952) on a Hewlett-Packard Model 30 calculator using program WBTA0109.

Pituitary and plasma samples

Adenohypophyses from adult Rana catesbeiana, Bufo bufo japonicus, Xenopus laevis and Hynobius tokyensis were individually homogenized in 0.5 ml of distilled water. Aliquots of the pituitary homogenates were used for the protein determination. Each homogenate was analyzed in the RIA. The blood of adult bullfrog was collected individually from the heart in a heparinized tube. Blood samples from stage X-XXII tadpoles and stage XXIII-XXV tadpoles were collected individually in heparinized hematocrit tubes from the artery of the tail and from the artery of the hind leg, respectively. Plasma was then obtained by centrifugation at 3,000 rpm for 15 min and stored frozen.

Results

In agar diffusion, a single precipitin line was observed between every antiserum obtained and fPRL or the bullfrog pituitary extract. Ovine PRL, bPRL, oGH and fGH did not react with the antiserum to fPRL (Fig. 1).

All antisera exhibited the ability to bind the labeled fPRL when approximately 0.2 ng 125I-fPRL was added to 500 μl of the diluted antiserum. The antiserum with the highest titre bound 35% of the label at a dilution of 1 : 50,000. About 70% of the labeled fPRL was precipitated by the excess antiserum (dilution of 1 : 500).

Administration of acetone-dried powder of bullfrog pituitary incubated with normal rabbit serum to premetamorphic tadpoles increased collagen synthesis in the tail fin. When the normal rabbit serum was substituted for the fPRL antiserum, the bioactivity of the pituitary powder was attenuated markedly. Injections of antiserum alone did not affect the collagen synthesis (Fig. 2).

The antiserum to fPRL reacted with the pituitary cells which were strongly erythrosine-positive when stained with Cleveland and Wolfe's trichrome. These cells were evenly scattered in a mid-sagittal section of the adenohypophysis (Fig. 3).

The fPRL standard gave a log dose inhibition of the binding of 125I-labeled fPRL to the fPRL antiserum (Fig. 4). The sensitivity of the PRL RIA, defined as 2SD below the 100% bound point, averaged 0.76±0.03 ng (Mean±SE) of fPRL standard per 100 μl assay buffer in 10 assays. Precision of the
Fig. 2. Inactivation of bioactivity of fPRL by fPRL antiserum. Stage X tadpoles received three injections of bullfrog anterior pituitary powder (AP) which had been incubated with the antiserum (A/S) or normal rabbit serum (NRS) for 24 hr. Incorporation of \(^{14}\)C-proline into the collagen fraction in the tail fin was determined as described in the Materials and Methods. Each histogram represents the mean of 5 determinations ±SEM. Statistical analysis was performed by analysis of variance. The value of NRS + AP was significantly different from those of other groups (p < 0.01).

Fig. 3. Two adjacent sections of bullfrog adenohypophysis. A, stained with Cleveland and Wolfe's trichrome. B, stained with peroxidase-labeled fPRL antiserum and Mayer's hematoxylin. The tissue was fixed in Bouin-Holland sublimate and cut sagittally at 4 μm in thickness. Cells reacted with fPRL antiserum correspond to strongly erythrosine-positive cells. Identical cells in A and B have the same number. (×800).
assay was determined by assaying the fPRL standard. The interassay coefficient of variation was 3.6% when the estimated dose at 50%-inhibition in 10 assays were employed. The intraasay coefficient of variation of 3.8% was obtained by the repeated determinations of 10 µg fPRL standard.

In Fig. 4, inhibition curves of the pituitary homogenates of *Rana catesbeiana*, *Bufo bufo japonicus*, *Xenopus laevis* and *Hynobius tokyoensis* are shown. The linear portion of each curve of *Rana* and *Bufo* pituitary homogenates was parallel to that of the fPRL standard. The dose-response curves of *Xenopus laevis* and *Hynobius tokyoensis* did not parallel that of the fPRL standard. Ovine PRL, bPRL, oGH and fGH showed no cross-reactivity in the fPRL RIA.

The slopes of inhibition produced by the plasma samples from the adult and larval (st. XXI) bullfrogs were parallel to that of the standard (Fig. 5). The PRL concentration was estimated from the linear portion of each curve. The PRL level in adult frog plasma ranged from 62 to 100 ng/ml. The plasma samples from the adult bullfrogs which had been hypophysectomized for 21 days had no detectable immunoreactive PRL.

Plasma PRL levels at various stages of development are shown in Fig. 6. During pre- and prometamorphosis, and at the onset of metamorphic climax, immunoreactive prolactin levels in the plasma were relatively low. At stage XXIII, when the tail length became one third of the body length, the PRL level rose suddenly and at stage XXIV the level reached a plateau, followed by a slight decline at stage XXV when the tail disappeared completely.
Fig. 5. Representative data from the RIA of fPRL. Purified fPRL standard (lower abscissa) was compared with plasma from intact and hypophysectomized frogs and pooled plasma from 6 tadpoles of stage XXI (upper abscissa). PRL concentrations of intact frog and tadpole plasma are estimated to be 78 and 35 ng fPRL per ml, respectively. Hypophysectomized frog plasma contained no detectable fPRL.

Fig. 6. Changes in plasma immunoreactive fPRL levels of bullfrog tadpoles at various stages of development. Plasma from 2-3 tadpoles of stage X-XXII were arbitrarily pooled and the 4-5 pooled samples per each stage were tested for PRL concentration. Plasma samples from individual animals of stage XXIII-XXV were tested separately. Data represent the mean±SEM.
Discussion

Anuran prolactin cells stain more readily with erythrosine than with orange G (van Oordt, 1974). This was confirmed by the present experiment since the pituitary cells which immunologically reacted with antisem to fPRL corresponded to the cells which were strongly erythrosine-positive.

The fPRL antiserum neutralized the collagen synthesis-stimulating activity of the pituitary powder markedly. It has been shown that most of the activity in the bullfrog pituitary gland derives from PRL (Kikuyama et al., 1980a).

Inactivation of the normally circulating PRL in premetamorphic tadpoles by the antiserum could not be detected by measuring collagen synthesis in the tail fin, although the antiserum completely blocked the elevation of collagen synthesis brought about by PRL which was released above the normal level by the administration of pimozide (Yamamoto and Kikuyama, 1982). The decline in bioactivity of the endogenous PRL brought about by the antiserum reflects the tail resorption in spontaneously metamorphosing tadpoles (Clemons and Nicoll, 1977b) and thyroxine-treated tadpoles (Yamamoto and Kikuyama, 1982). The antiserum-injected specimens lost their tail more rapidly than the normal rabbit serum-injected tadpoles. These results indicate that the endogenous PRL acts at least as an antimetamorphic hormone.

Among the works in which thyroid hormone levels of bullfrogs during metamorphosis were measured, most of the results indicate that the hormone level begins to rise in the late premetamorphic stage and reaches a plateau by stage XXIII (Miyauichi et al., 1977; Regard et al., 1978; Mondou and Kaltenbach, 1979; Suzuki and Suzuki, 1981). Etkin (1968) predicted that PRL levels are relatively low and thyroid hormone levels are relatively high during the premetamorphic and early premetamorphic periods and that PRL levels decline and thyroid hormone levels are elevated as metamorphosis progresses, so that at the climax stage thyroid hormone-induced metamorphic changes occur rapidly.

According to our data obtained here, there was no significant decline in the prolactin level at late prometamorphic and early climax stages when compared with the level at preceding stages. Assuming that the amount of immunoreactive prolactin is an indication of prolactin bioactivity, the antimetamorphic action of prolactin must be overcome by thyroid hormone.

In this respect, adrenal steroids, of which synthesis (Hsu et al., 1980) and release (Dale, 1962; Jaffe, 1981) is enhanced around the onset of metamorphic climax, may play an important role in completing metamorphosis. Earlier reports indicate that thyroid hormone-induced metamorphosis is accelerated by corticoids (Frieden and Naile, 1955; Kaltenbach, 1958; Kobayashi, 1958). In Bufo and Rana, adrenocortical tissue principally secretes two hormones, aldosterone and corticosterone (Carstensen et al., 1961; Crabbe, 1961). In the presence of aldosterone or corticosterone, a subthreshold dose of thyroid hormones induced a remarkable shrinkage of isolated tadpole tails in vitro (Kikuyama et al., 1981). Recently, we have obtained data suggesting that aldosterone augments thyroid hormone action by increasing a maximal nuclear binding capacity for thyroid hormones in the tadpole tail (Niki et al., 1981).

Around stage XXIV, the immunoreactive PRL level rose markedly. This coincides closely with the results obtained by Clemons and Nicoll (1977a). PRL released from the pituitary gland at this stage may hardly act as an antimetamorphic hormone since the tissues have been subjected to the high concentration of thyroid hormones and have undergone a considerable transformation.

RIA of PRL in the pituitary glands of several amphibians revealed that PRL of Bufo possesses immunological properties similar to
those of fPRL. It is of interest that characteristics of PRL of *Xenopus* and *Hynobius* are somewhat different from those of fPRL.

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**References**


