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Effects of Gonadotropin-Specific Antibodies on the Interaction of Follicle-Stimulating Hormone and Luteinizing Hormone with Testicular Receptors in the Bullfrog, *Rana catesbeiana*

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ABSTRACT—To study the properties of the receptor-binding site in molecules of bullfrog gonadotropins, we treated follicle-stimulating hormone (fFSH) and luteinizing hormone (fLH) from the bullfrog with antibodies raised against the whole molecules or subunits of these hormones, and then examined the binding of the treated hormones to bullfrog testicular receptors. When low concentrations of fFSH-specific and fLH-specific antisera were used, increased concentrations of the antisera led to increased inhibition of binding of both hormones. At higher concentrations, however, the extent of binding was elevated by the antisera in both cases. These stimulatory effects were abolished by treatment of antisera with papain, while the inhibitory effects persisted. Binding of fFSH to its receptors was inhibited by a monoclonal antibody against fFSH-β and by one against fFSH-α but not by a monoclonal antibody against fLH-β. The inhibitory potency of the antibody against fFSH-α was stronger than that of the antibody against fFSH-β. Binding of fLH was inhibited by the monoclonal antibody against fLH-β more strongly than by one against fFSH-α. Binding of fLH was not inhibited or slightly inhibited by the monoclonal antibody against fFSH-β. These results suggest that both the α and β subunits contribute to the binding of both hormones to their receptors. Although fFSH and fLH bind to a common receptor in the bullfrog testis, the chemical structures of the receptor-binding sites in these hormones are assumed not to be identical. The importance of the α subunit in the binding of fFSH to receptors is also suggested.

INTRODUCTION

The pituitary gland of anuran amphibians, as well as that of higher vertebrates, secretes two species of gonadotropins, i.e. follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These hormones have been reported to exert different effects in the frog testis [1–4]. However, Takada *et al.* [5] found that specific binding of ^{125}I-fFSH was completely inhibited by both fFSH and fLH. Recently, we further showed that specific binding of ^{125}I-fLH was similarly inhibited by both fFSH and fLH, although the inhibition curves were not parallel for these two hormones [6]. These results suggest the close similarity or identity of the chemical structures of the receptor-binding sites in the fFSH and fLH molecules.

Antibodies, raised against intact molecules or subunits of gonadotropins, have been employed as useful tools for the analysis of the interactions between gonadotropins and their receptors in mammals [7–17]. Using antibodies raised against fFSH, fLH and their subunits, we examined the effects of the antibodies on binding of fFSH and fLH to testicular receptors of the bullfrog, in order to analyze the interactions between bullfrog gonadotropins and their receptors, with special emphasis on the loss or incompleteness of FSH/LH specificity of the receptors.

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MATERIALS AND METHODS

Tissues

Fresh testes of adult bullfrogs, *Rana catesbeiana,*

were collected at the time of meat processing at the shop of a vendor of frog meat. The testes were immediately put in a chilled solution of 0.68% NaCl and were transported under chilled conditions to the laboratory.

Hormones, antisera and monoclonal antibodies

Highly purified fFSH and fLH, designated FF1341B and FL461B by Takada and Ishii [4], respectively, were used.

Two types of antiserum were used: one was an antiserum raised against fLH, designated No. 1 by Takada and Ishii (unpublished) and the other was an antiserum against fFSH designated No. 3.

Three types (BF3A20, BF3B25, and BL4B11) of monoclonal antibody against subunits of gonadotropin were used. They were raised against the fFSH-α, fFSH-β and fLH-β subunits, respectively. The first two were prepared by Tanaka *et al.* [18] and the last by Park *et al.* [19].

In addition, a monoclonal antibody against mammalian luteinizing hormone-releasing hormone (LHRH), HAC-MM02-MSM84 of Park and Wakabayashi [20], was used. All these monoclonal antibodies were supplied by Prof. K. Wakabayashi of Gunma University.

Goat anti-rabbit immunoglobulin was supplied by Dr. T. Yoshida of Tsukuba Primate Center, and goat anti-mouse immunoglobulin (IgA+IgG +IgM) was purchased from Cappel (West Chester, PA., USA). They were used as second antisera to precipitate antibody-bound hormones.

Chemicals

Insoluble papain was obtained from Sigma (St. Louis, MO., USA; lot 24F-9620; 850 units/g). $Na^{125}I$ was obtained from ICN biochemicals (Irvine, CA., USA). All other reagents were purchased from standard commercial sources.

Radioiodination

Bullfrog FSH and LH were radioiodinated with ^{125}I by the lactoperoxidase method reported by Takada *et al.* [5]. Specific radioactivities, estimated by the method of Kubokawa and Ishii [21], of ^{125}I-FSH and ^{125}I-LH were about 85,000 and 90,000 cpm/ng (64 and 68 μCi/μg), respectively.

Digestion of antisera

Digestion of antisera with papain was performed by the method of Dias *et al.* [10], after appropriate dilution of the antisera. A suspension of enzyme was prepared first by incubating 12 mg of insolubilized papain with 1.14 ml of an activating solution (a mixture of deionized water, 0.01 M EDTA, 0.006 M mercaptoethanol, and 0.05 M cysteine-HCl in a ratio of 70 : 10 : 1 : 10) for 30 min. To 1.5 ml of each solution of antiserum, 125 μl of the enzyme suspension were added. Digestion was allowed to proceed for 30 min at 37°C in a shaking bath. After the incubation, 20 μl of 0.1 M dithiothreitol were added. The mixture was then centrifuged to remove insoluble papain. The supernatant was dialyzed overnight against 40 mM Tris buffer. The dialyzate was used as papain digested monovalent antiserum in binding experiments.

Preparation of receptors

A crude fraction of plasma membranes was prepared from bullfrog testis by the method previously described [6], and it was used as the preparation of receptors for binding experiments. An aliquot of 0.1 ml of the final suspension of membranes contained membrane particles derived from 50 to 100 mg of testicular tissue.

Binding experiments

Various dilutions of each antibody or buffer alone (50 μl) were preincubated with 50 μl of ^{125}I-fFSH or ^{125}I-fLH (about 0.2 to 0.4 ng or 18,000 to 32,000 cpm) in plastic tubes at 20°C for 3.5 hr. To each tube, 100 μl of the preparation of receptors were added after the preincubation. Then tubes were incubated at 20°C for 3 hr with shaking. At the end of the incubation, 1 ml of cold buffer was added to each tube, and the tubes were centrifuged at 10,000$\times g$ for 3 min at 4°C. The supernatant was removed by aspiration, and the pellet was resuspended in buffer and centrifuged again. Radioactivity of the resulting pellet was measured in an automatic gamma counter. The hormone-binding level was expressed as a percentage of the radioactivity bound in the absence of antibody. The level of nonspecific binding

estimated in the absence of the antibody but in the presence of excess nonradioiodinated gonadotropins was 9 and 19% of the total binding for fFSH and fLH, respectively.

In order to study immunological cross-reactivity and potency of antibodies, we used the following procedures. An antibody and labeled hormone were incubated under conditions similar to those in the preincubation described for receptor-binding experiments, and then 200 μl of appropriately diluted second antiserum was added. Reaction tubes were then incubated at 20°C for 5 hr. After addition of 500 μl of 1% BSA-PBS, the tubes were centrifuged at 3000×g for 40 min, and radioactivity in the resulting pellets was measured.

RESULTS

Effects of polyclonal antisera against fFSH and against fLH on binding of radioiodinated fFSH and fLH to testicular membrane receptors

The antiserum against fFSH inhibited the binding of ^{125}I-fFSH to the receptors in proportion to the concentration of the antiserum at low concentrations of the antiserum (Fig. 1a). However, at higher concentrations, the extent of the inhibition became smaller as the concentration of the antiserum became higher, and augmentation of the binding of ^{125}I-fFSH above the 100% level was observed at the highest concentration tested.

The antiserum against fLH also inhibited the binding of ^{125}I-fFSH but, in this case, no augmentation above the 100% level was observed

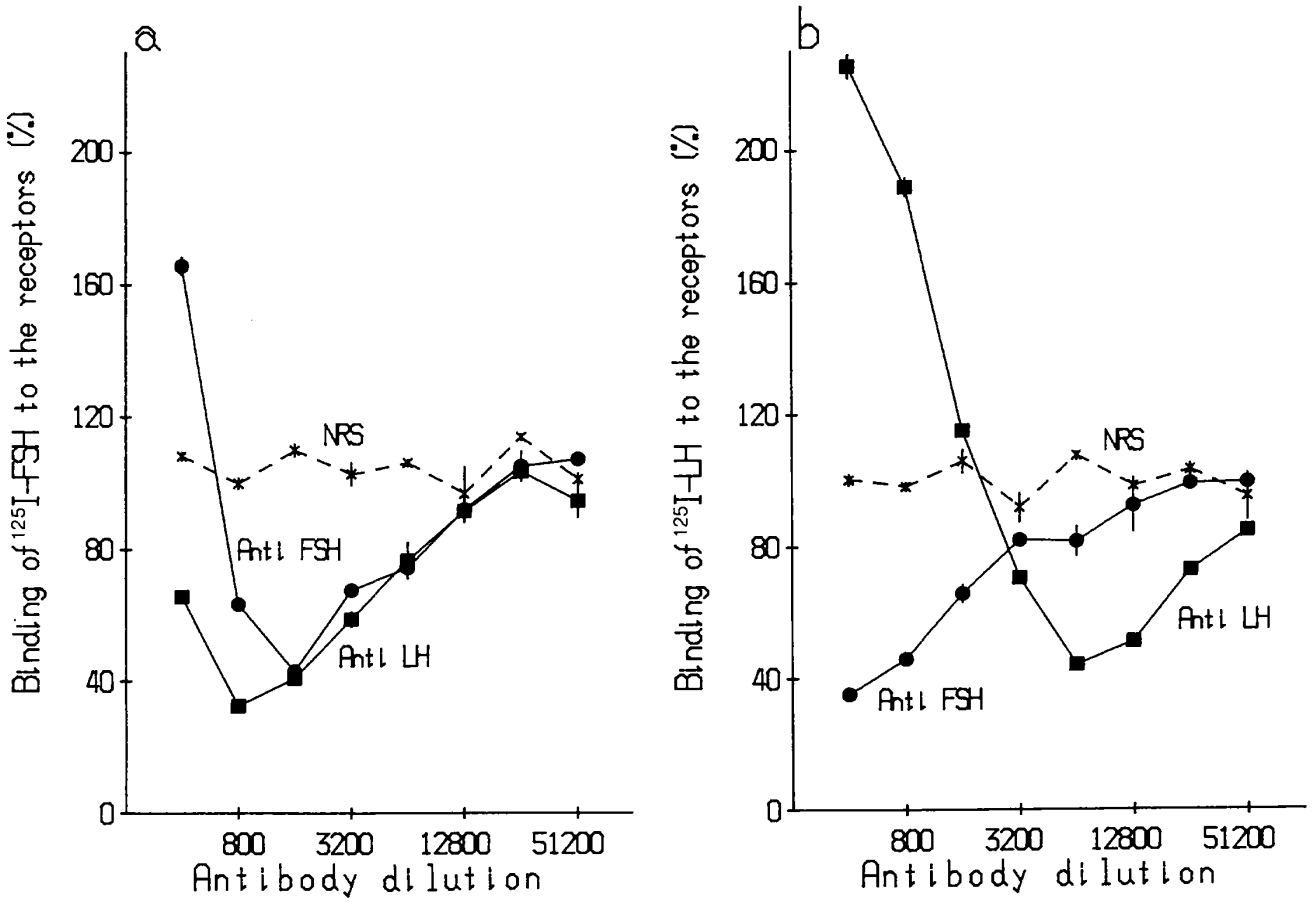

FIG. 1. Effects of a polyclonal antiserum against fFSH (●, solid line), a polyclonal antiserum against fLH (■, solid line) and normal rabbit serum (×, dotted line) on binding of ^{125}I-fFSH (a) and ^{125}I-fLH (b) to a preparation of bullfrog testicular receptors at different dilutions. The ordinate shows the binding of radioiodinated hormone as a percentage of radioactivity bound in the absence of antiserum. Each point and each vertical bar show the mean and the range of duplicate determinations, respectively. See text for details.

although the extent of inhibition became smaller at the highest concentration of antiserum tested in this antigen excess condition.

The antiserum against fLH strongly inhibited the binding of ^{125}I-fLH, with the extent of inihbition depending on the concentration of antiserum at lower concentrations of antiserum (Fig. 1b). At higher concentration, the inhibitory effect of the antiserum became smaller as the concentration was increased, and the binding of ^{125}I-fLH exceeded the 100% level at the three highest concentrations tested. Antiserum against fFSH inhibited the binding of ^{125}I-fLH, with the extent of inhibition depending on the concentration over the whole range of concentrations tested. However, the inhibitory potency of the antiserum against fFSH was lower than that of the antiserum against fLH. The normal rabbit serum (NRS) showed no effect on binding of either ^{125}I-fFSH or ^{125}I-fLH at any concentration of the serum tested.

Effects of papain-digested antisera on binding of fFSH and fLH to testicular receptors

After digestion with papain, both the antiserum against fFSH and that against fLH inhibited binding of ^{125}I-fFSH to the receptors, with inhibition depending on the concentration over almost the entire range of concentrations tested (Fig. 2a). No augmentation was observed at all with either treated antiserum. The potency of the inhibition of binding of the digested antiserum against fFSH was 1.7 times higher than that of the digested antiserum against fLH at the 50% level of inhibition.

Binding of ^{125}I-fLH was also inhibited by both treated antisera in a concentration-dependent manner, and absolutely no augmentation was observed (Fig. 2b). The inhibitory potency of the

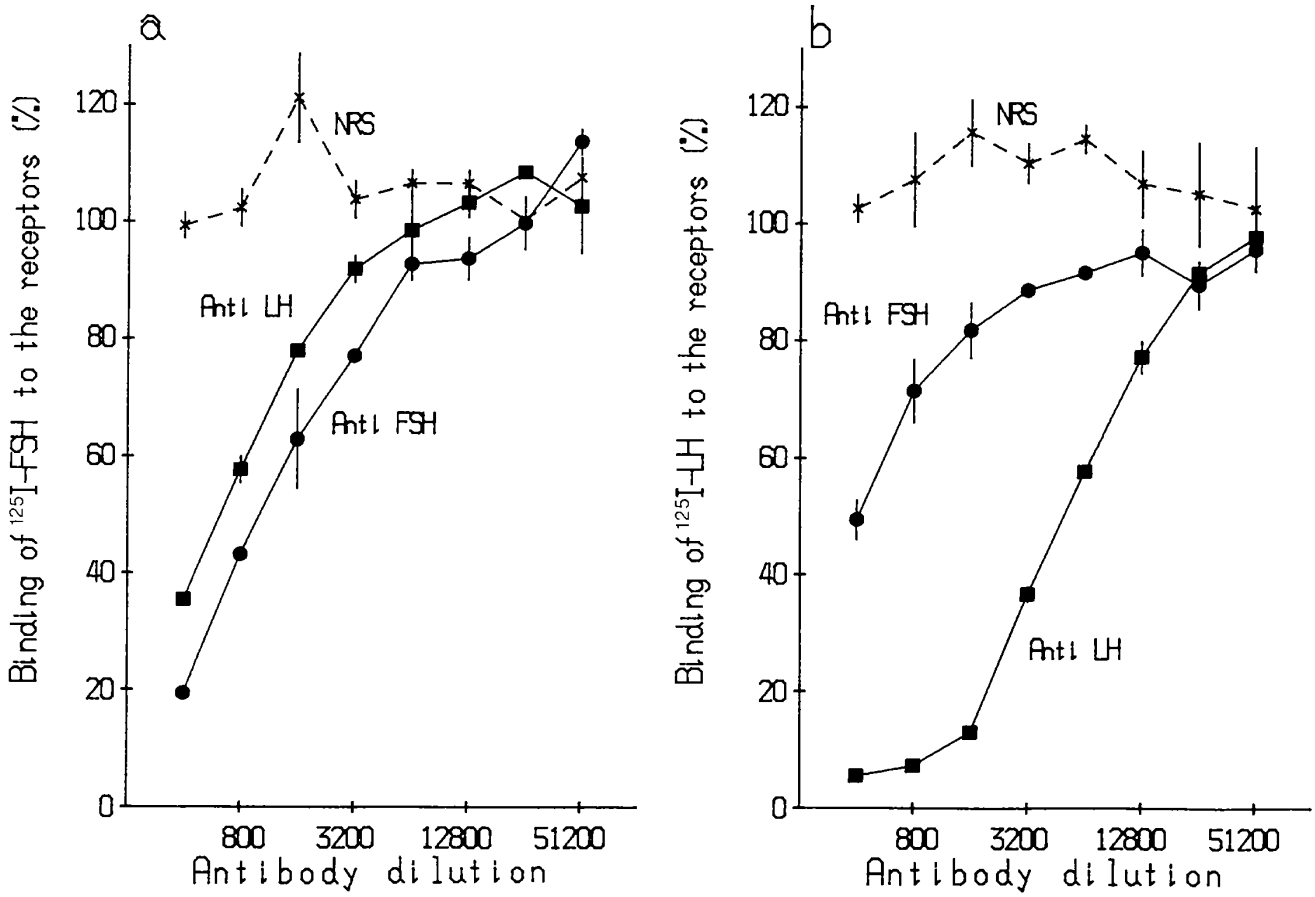

FIG. 2. Effects of a papain-digested antiserum against fFSH (●, solid line), a papain-digested antiserum against fLH (■, solid line) and papain-digested normal rabbit serum (×, dotted line) on the binding of ^{125}I-fFSH (a) and ^{125}I-fLH (b) to the preparation of bullfrog testicular receptors at different dilutions. See the legend to Figure 1. and text for details.

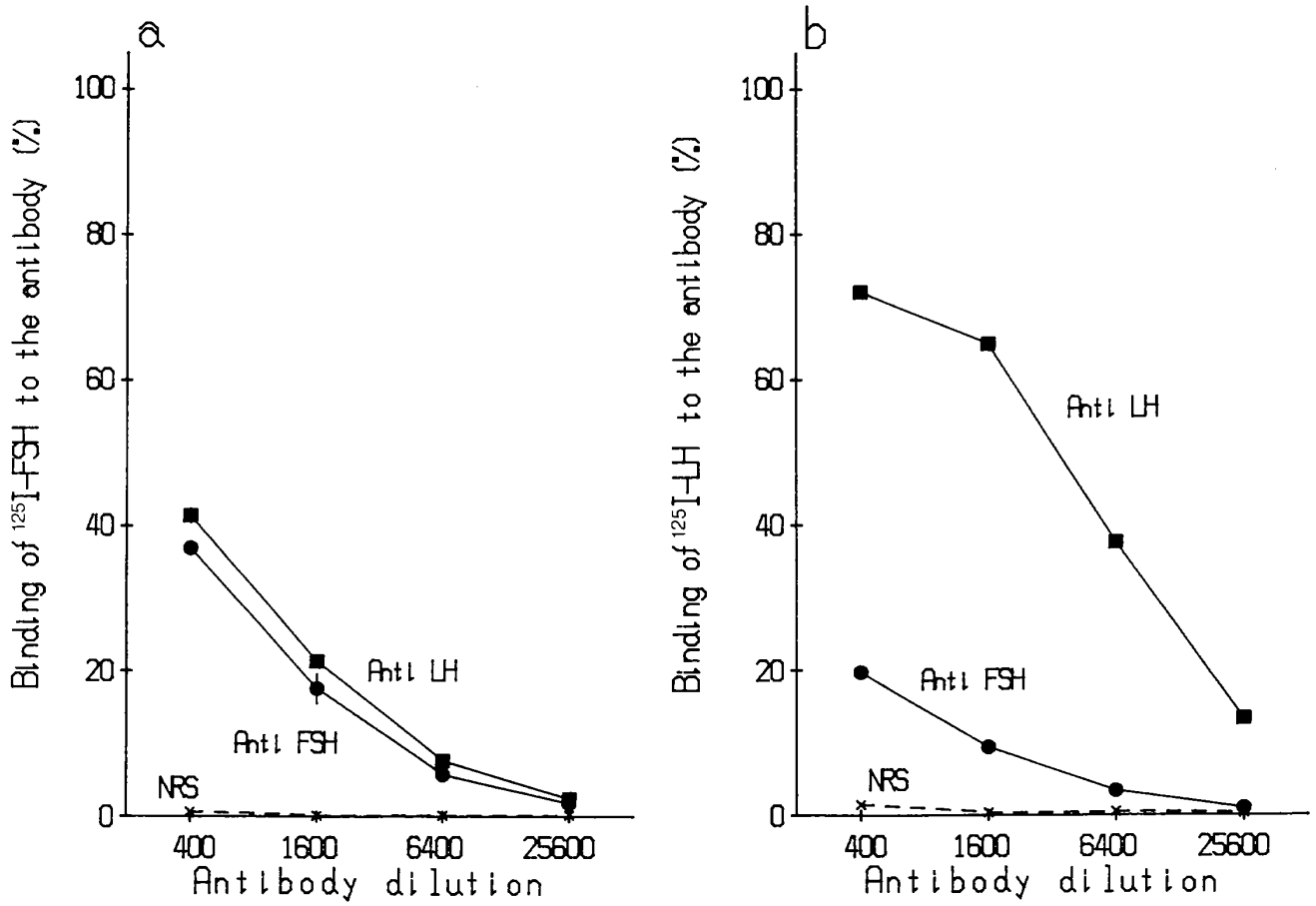

FIG. 3. Binding of ^{125}I-fFSH (a) and ^{125}I-fLH (b) to different dilutions of a papain-digested antiserum against fFSH (●, solid line), papain-digested antiserum against fLH (■, solid line) and papain-digested normal rabbit serum (×, dotted line). The ordinate shows the proportion of antibody that bound radioiodinated hormone as a percentage of the amount of added hormone. Each point is the mean and each vertical bar shows the range of duplicate determinations. See text for details.

digested antiserum against fLH was far higher (12.6 times higher at the 50% level of inhibition) than that of the digested antiserum against fFSH.

The FSH/LH specificity and immunoreactive potency of the antiserum against fFSH and fLH were studied with the papain-digested antisera (Fig. 3a, b). Proportions of ^{125}I-fFSH bound to papain-digested antiserum against fFSH and to antiserum against fLH were similar, being about 40% at a dilution of 1:400. In contrast, the antiserum against fLH bound ^{125}I-fLH more strongly (about 70% binding at a dilution of 1:400) than the antiserum against fFSH (20% binding at the same dilution).

Effects of monoclonal antibodies on binding of fFSH and fLH to testicular receptors

Binding of ^{125}I-fFSH to the receptors was inhibited by both the monoclonal antibodies raised against fFSH-β and against fFSH-α, but not by that against fLH-β (Fig. 4a). The monoclonal antibody against fFSH-α was more potent than that against fFSH-β in inhibition of the binding of ^{125}I-fFSH to the receptors. The ratio of the inhibitory potencies, calculated with 100- and 400-time dilution data, was about 2.8:1.

Binding of ^{125}I-fLH to the receptors was also inhbited by both the monoclonal antibody against fLH-β and that against fFSH-α. The inhibitory potency of the former antibody was far higher than that of the latter. The potency of the former exceeded that of the latter 60 fold. No significant inhibition of binding of ^{125}I-fLH to its receptors by the monoclonal antibody against fFSH-β was observed (Fig. 4b).

A monoclonal antibody against mammalian

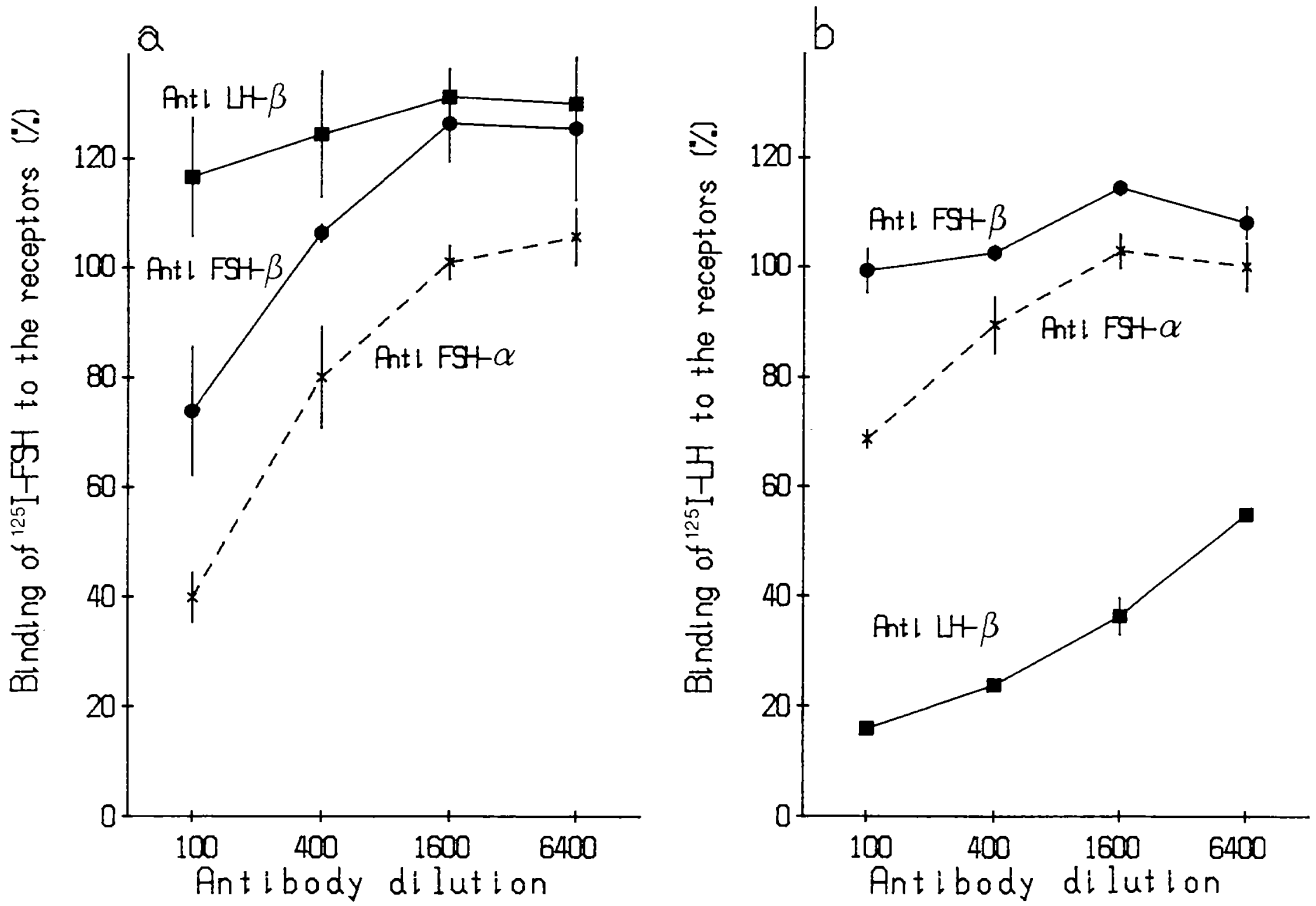

FIG. 4. Effects of monoclonal antibodies against fFSH-α (×, dotted line), against fFSH-β (●, solid line) and against fLH-β (■,, solid line) on binding of ^{125}I-fFSH (a) and ^{125}I-fLH (b) to a preparation of bullfrog testicular receptors at different dilutions. See the legend to Fig. 1 and text for details.

LHRH did not affect the binding of either ^{125}I-fFSH or ^{125}I-fLH to the receptors (data not shown).

The FSH/LH specificity and immunological potencies of the monoclonal antibodies are shown in Figures 5a and b. The extent of binding of ^{125}I-fFSH to the monoclonal antibody against fFSH-β was about 32% at a dilution of the antibody of 1 : 100, while the extent of binding of ^{125}I-fFSH to the monoclonal antibody against fFSH-α was only 12% at same dilution of antibodies.

The extent of binding of ^{125}I-fLH to the monoclonal antibody against fLH-β reached about 62% at a dilution of the antibody of 1 : 100 and that of ^{125}I-fLH to the monoclonal antibody against fFSH-α was only about 10%. No apparent binding of ^{125}I-fFSH to the monoclonal antibody against fLH-β or of ^{125}I-fLH to the monoclonal antibody against fFSH-β was observed.

DISCUSSION

We showed in the present study that both of the polyclonal antisera against fFSH and fLH inhibited the binding of both fFSH and fLH to their receptors at low concentrations of antiserum. However, at higher concentrations, the antisera became less effective in inhibiting the binding or even enhanced the binding to receptors. Dias *et al.* [10] also reported that antisera against subunits of human FSH enhanced binding of human FSH to its receptor, and they suggested that the enhancement of the binding might be caused by formation of aggregates of antibody and hormone molecules due to bivalency of the antibody molecule. They were able to eliminate the enhancement of binding by pretreating the antisera with papain, which digests bivalent molecules of the antibody and produces monovalent molecules. In the present investigation, the pretreatment of antisera against

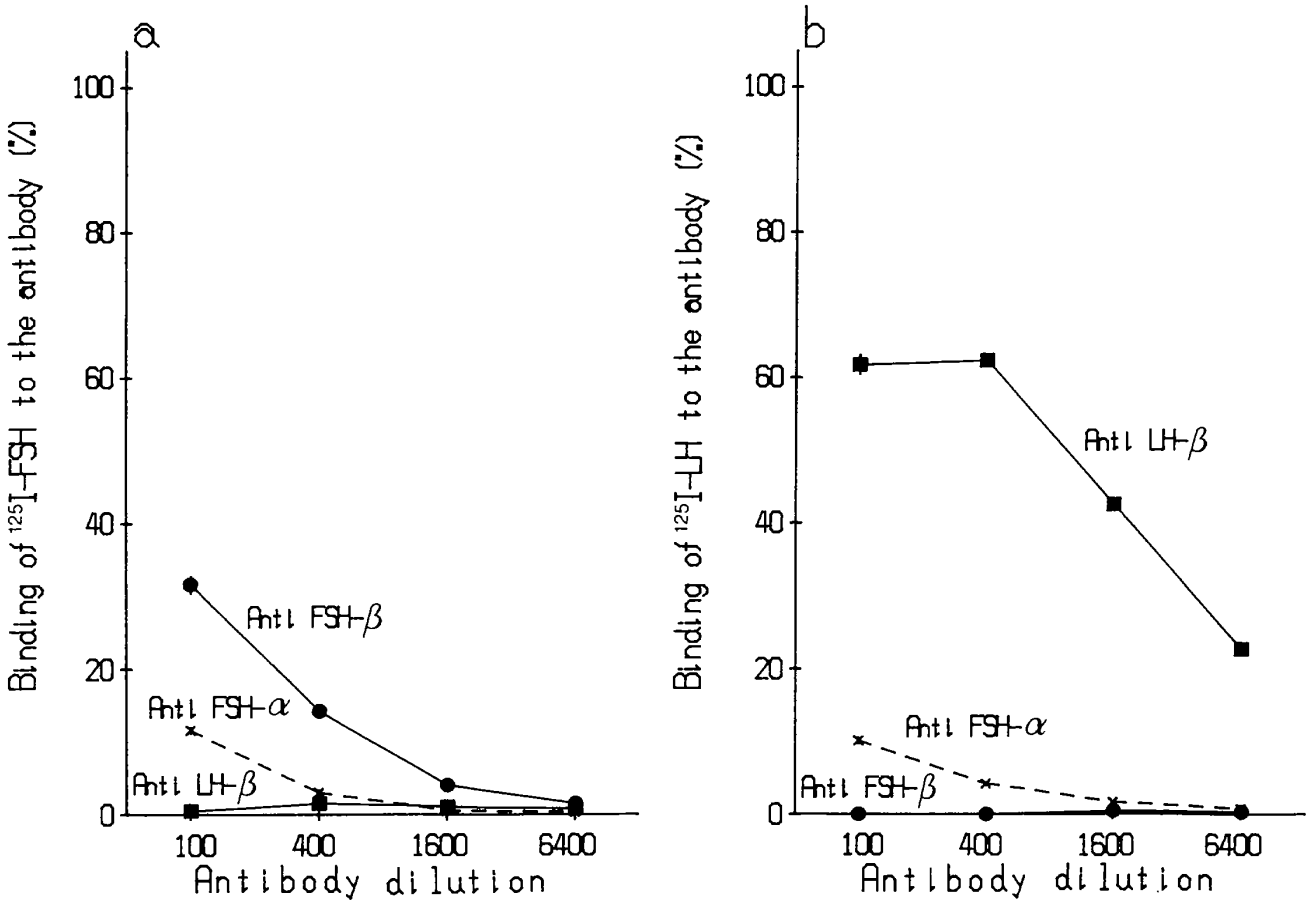

FIG. 5. Binding of ^{125}I-fFSH (a) and ^{125}I-fLH (b) to different dilutions of monoclonal antibodies against fFSH-α (×, dotted line), against fFSH-β (●, solid line) and against fLH-β (■,, solid line). See the legend to Fig. 3 and text for details.

fFSH and fLH with papain completely eliminated the enhancement of the binding to receptors observed at high concentrations of the antisera. Consequently, the enhancement of the binding to receptors by the antisera, observed in the present study, is also considered to be caused by the formation of aggregates that results from the bivalency of the antibodies.

The antiserum against fFSH was more potent than the antiserum against fLH in inhibiting the binding of ^{125}I-fFSH, while the former was less potent than the latter in terms of immunological potency for binding fFSH. The ratio of the inhibitory potencies in binding to receptors was 1.7 : 1 and the ratio of the immunological potencies was 1 : 1.2. The antiserum against fLH was more potent than the antiserum against fFSH in terms of both inhibition of the binding of ^{125}I-fLH to receptors and in terms of the immunological capacity to bind fLH, although the potency ratios differed significantly between these two parameters (13 : 1 in the inhibition of receptor-binding and 44 : 1 in the immunological binding). These results imply that some components of these antisera inhibit the binding to receptors by binding to the hormone molecule, but the other components do not, and also that the proportion of these two types of component differs between antiserum against fFSH and that against fLH.

When monoclonal antibodies were used, that against fLH-β inhibited the binding to receptors of only fLH and not fFSH, and that against FSH-β inhibited binding of only fFSH and not fLH. These results seem to conflict with the fact that both fFSH and fLH bind to a common receptor site in the bullfrog testis, since specific antibodies to a unique portion of each hormone molecule inhibit the binding of the hormones to the common receptor site. However, we can explain these results by assuming that the sites which are related

to the receptor binding in the fFSH and fLH molecules have at least somewhat different chemical structures, and our monoclonal antibodies against fFSH-β and fLH-β bound to these specific or unique parts. In the case of mammalian LH and hCG, a similar result was reported by Schwarz *et al.* [16]. They found that monoclonal antibodies specific to human LH and to hCG inhibited binding of these hormones to the common testicular receptor in the rat. Hattori *et al.* [17] reported that the conformation of the β subunit of deglycosylated hCG was altered by the binding of antibody against hCG-β. It is possible that the binding of antibody causes the conformational change of receptor binding site in hormone molecule.

Unlike the antibodies against fLH-β and fFSH-β, the antibody against fFSH-α inhibited the binding to receptors of both hormones, although the binding of fFSH was more strongly inhibited than that of fLH. This result suggests that masking of the α subunit with antibodies influences the hormone-receptor interaction, and it seems to conflict with the idea that the α subunit is involved in the action of the hormone while the β subunit is involved in the specificity of the hormone [22–25]. However, a contribution of not only the β subunit but also of the α subunit to the direct interaction of the hormone and receptor has been reported by several investigators: Milium and Midgley [11] in the case of binding of hCG to the ovarian receptor of the rat; Dias *et al.* [10] in the case of binding of human FSH to the testicular receptor of the calf; Moyle *et al.* [8] in the case of binding of hCG to the Leydig cell receptor of the rat; and Schwarz *et al.* [16] in the case of binding of hCG and human LH to the testicular receptor of the rat.

In the inhibition of binding of fLH, the antibody against fFSH-α was less potent than the antibody against fLH-β. However, in inhibition of binding of fFSH, the relationship was reversed. The potency of antibody against the fFSH-α subunit was higher than that of antibody against fFSH-β, although their immunological potencies were reversed. This result suggests the importance of the α subunit in the binding of fFSH to receptors, in particular, in the bullfrog.

As mentioned in the "Introduction", the same gonadotropin receptor in the bullfrog testis recognizes both fFSH and fLH, although in mammals the two hormones have separate receptors. Accordingly, the importance of the α subunit in the hormone-receptor interactions of both fFSH and fLH in the bullfrog, as revealed in the present study, can be related to the absence of FSH/LH specificity in the bullfrog testicular gonadotropin receptor. However, this phenomenon is not unique to the bullfrog. Dias *et al.* [10] reported that the binding of human FSH (hFSH) to the calf testicular receptor was inhibited by an antiserum against hFSH-α more strongly than by an antiserum against hFSH-β. Therefore, we can not simply relate our result on the inhibitory effect of the antibody against fFSH-α on the binding to receptors to lack of FSH/LH specificity in the bullfrog gonadotropin receptor.

This is the first report of a study of the interaction of subunits of FSH and LH with their receptor using monoclonal antibodies in nonmammalian vertebrates. Even in the mammal, there is only one report to our knowledge, on the inhibition of the binding of FSH to receptors by a monoclonal antibody [13]. Further studies using various types of monoclonal antibody are needed to elucidate the consequences of the lack of FSH/LH specificity in the binding to receptors in amphibians.

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