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Estradiol Induces and Hyperglycosylates the Receptor for Ovine Gonadotropin-Releasing Hormone*

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ABSTRACT

The crucial first link between GnRH and its pleiotropic stimulation of the reproductive system is its receptor (GnRHRec). In mammals, 17β-estradiol is a major regulator of GnRH action, and part of its regulation occurs at the level of the GnRHRec. In ovine pituitary cultures, estradiol simultaneously increases GnRHRec and GnRH-stimulated LH secretion (the LH response), but after 6–15 h the effect of estradiol becomes paradoxical, and the LH response rapidly decreases to control levels (by 24 h), whereas GnRHRec remains elevated. A preliminary study used photoaffinity labeling of the GnRHRec to show that estradiol can induce 38- and 43-kDa GnRHRec. The photoaffinity technique has been used here to 1) further investigate estradiol-mediated induction of GnRHRec, 2) define the nature of the different sized GnRHRecs, and 3) determine whether the larger size is related to degradation of the LH response. The effect of estradiol is compared with that of inhibin, which only induces the 38-kDa GnRHRec and always increases the LH response to GnRH treatment. Receptors for GnRH in ovine pituitary cultures were photoaffinity labeled with [^{125}I](azidobenzoyl-D-Lys^{6}-des-Gly^{10})-GnRH-*N*-ethylamide and analyzed by SDS-PAGE. Treatment with estradiol or inhibin for 6–24 h induced a 38-kDa GnRHRec only. Further treatment with estradiol (>24 h), but not inhibin, shifted the apparent M_r of the GnRHRec to 43 kDa. Phosphatase treatment did not reverse this apparent M_r change. Analysis of receptor glycosylation using *N*-glycosidase F or tunicamycin showed that the 43-kDa GnRHRec was a hyperglycosylated form of the 38-kDa GnRHRec. The 38-kDa GnRHRec, in turn, was a glycosylated form of the 29-kDa GnRHRec. The studies presented here define several glycosylated intermediates of the ovine GnRHRec that are induced by estradiol and/or inhibin. The function of estrogen-mediated hyperglycosylation is unclear, but kinetic studies dissociate it from degeneration of the LH response to GnRH. (*Endocrinology* **141:** 91–99, 2000)

GnRH IS ESSENTIAL for reproduction in vertebrates (1). It activates pituitary gonadotropes via a G protein-coupled receptor (GnRHRec) (2), resulting in the synthesis and release of LH and FSH, which are the two gonadotropins in mammals. The gonads, stimulated by FSH and LH, then produce hormones such as estradiol and inhibin that feed back to control the ability of GnRH to stimulate LH and FSH synthesis/release. A significant amount of this feedback regulation occurs directly at the GnRHRec level.

Estradiol can increase GnRH binding in sheep pituitary cultures (3–5) and in sheep pituitaries *in vivo*, thereby helping to generate a robust preovulatory LH surge (6–10); a similar phenomenon is documented for the rat (11, 12). In cultures of ovine pituitary cells, estradiol more than quadruples all three sizes of GnRHRec messenger RNA (mRNA) (13) as well as GnRH binding (3–5) within 6–15 h. GnRH-stimulated LH secretion (referred to below as the LH response) also increases 2- to 3-fold during the first 6–15 h of estradiol treatment, as measured after a standard treatment with 10 nM GnRH (3, 14). The increase in both GnRH binding and the LH response seem causally linked. However, by 24 h, the LH response often falls to control levels or lower, even though GnRH binding still remains high (3, 14). As the rapid increase in GnRH binding and responsiveness to GnRH may help initiate the preovulatory LH surge in sheep, degradation of the LH response after 6–15 h of estradiol treatment may help terminate it as well as serve other important functions involving gonadotropin synthesis. For these reasons, it is important to understand what causes the decrease in GnRH-stimulated LH secretion after extended treatment with estradiol.

Preliminary studies with photoaffinity labeling of the GnRHRec (15) showed that 36 h of estradiol treatment induced a 38-kDa GnRHRec and a larger GnRHRec near 43 kDa (16). The larger form of GnRHRec stood out in these experiments, as parallel treatments with inhibin induced only the lower M_r form (38 kDa). Previous studies with inhibin had shown that inhibin increases all three forms of GnRHRec mRNA (13), GnRH binding (4, 17), and GnRH-stimulated LH secretion (14, 17) just like estradiol, but the LH response never decreases or degrades even after 72 h of inhibin treatment. As estradiol uniquely induced both the 43-kDa GnRHRec and desensitization of the LH response, it was of interest to know whether these two phenomena were causally linked.

Desensitization of G protein-coupled receptors is often mediated by phosphorylation (18). As protein phosphorylation often causes a significant increase in apparent M_r (19, 20), it was thought that estradiol might desensitize the GnRHRec by causing it to be phosphorylated. Alternatively, the shift in apparent M_r from 38 to 43 kDa could reflect differential translation of the multiple mRNA species encoding the GnRHRec (13), which might give the appearance of desensitization but really reflect replacement of one GnRHRec with another that had different regulatory functions. Changes in M_r could also result from altered glycosylation,

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which is a process that can affect the efficiency of transport of G protein-coupled receptors to the plasma membrane as well as ligand binding (21). It was thought important to investigate the nature of the GnRHRec size shift to understand its potential for regulating GnRH action.

Materials and Methods

Ovine pituitary cell culture

Pituitaries were obtained from cross-bred ewes in the estrous season (September through April). Ewes were killed by a method approved by the institutional animal care and use committee of North Carolina State University, and pituitaries were removed within 5 min of death. Pituitary cells were dispersed using collagenase and pancreatin as reported previously (22) with yields of 60–80% based on 300 million cells/g tissue. Dispersed cells were plated in 100- or 150-mm plates at a concentration of 0.2 million/cm^2 and cultured under 95% air-5% CO_2 in medium 199 containing 10% charcoal-treated sheep serum, 25 mM HEPES buffer, and antibiotics as previously reported (22). Cultures were permitted to rest for 2 days before hormone treatments began. When the effect of estradiol on GnRH-stimulated LH secretion was studied, cells were preincubated with estradiol for specific time periods (0, 6, 12, 24, 48, and 72 h) and then treated with 10 nM GnRH for 6 h. GnRH treatment was begun in all cultures at the same time so that the age of all cultures was identical for all treatments. Media were collected and frozen at −20 C before assay for LH. When cells were used for GnRHRec analysis, they were treated with estradiol and/or inhibin for specific time periods (0, 6, 12, 24, 36, 48, 60, and 72 h), then harvested by scraping with a plastic spatula and centrifuged at 500 × *g*, and the pellet was frozen immediately in liquid nitrogen and stored at −80 C before being assayed by photoaffinity labeling with [^{125}I]GnRHA (^{125}I-labeled D-Lys^6-des-Gly^{10}GnRH-*N*-ethylamide). Cells were all scraped at the same time so that the ages of all cultures were identical for all treatments.

Assay for LH

LH was assayed by RIA according to published procedures (3, 17).

αT3-1 cells

αT3-1 cells were provided by Dr. P. Mellon (University of California, San Diego, CA) (23). These cells were grown at 37 C in DMEM (Life Technologies, Inc., Grand Island, NY) containing 10% FBS (HyClone Laboratories, Inc., Logan, UT) under 95% air-5% CO_2. Cells were grown in 150-cm^2 tissue culture flasks until confluent, harvested, pelleted at 1000 × *g*, and frozen at −80 C until use. The pellets were thawed, resuspended in binding buffer, and photolabeled with [^{125}I]azido-GnRHA.

17β-Estradiol and inhibin preparations

17β-Estradiol (Sigma, St. Louis, MO) was used to treat cultured cells at a concentration of 10 nM. Although this concentration of estradiol is hyperphysiological, it gave rapid, reliable responses that were not different from those obtained with 0.1 nM estradiol. Estradiol was dissolved in 95% ethanol (2 μM stock solution) so that concentrations of ethanol never exceeded 0.5% in culture media. This concentration of ethanol has never altered gonadotrope function in ovine pituitary culture. Two types of inhibin preparations were used. In Figs. 2. and 3, an enriched preparation of porcine follicular inhibin was used. Based on its potency, the inhibin preparation appeared to be 0.1% pure (assuming an ED_{50} of 1 ng/ml for pure 32-kDa inhibin); it was the inhibin preparation used in all past studies from this laboratory (3, 4, 13, 17). Purified recombinant human inhibin A was obtained as a gift from Dr. A. Mason (Prince Henry's Institute of Medical Research, Monash Medical Center, Victoria, Australia) (24) and was used to obtain the data shown in Fig. 4. The effects of both inhibin preparations were identical.

Radioiodination and azido-derivitazation of GnRHA

GnRHA (dLys^{6}desGly^{10}GnRH ethylamide), a superagonist analog of GnRH, was initially a gift from Dr. N. Ling (The Salk Institute, La Jolla, CA; at the time of the gift) and later purchased from Bachem (Torrance, CA). Twenty-five micrograms of GnRHA were radioiodinated using the Iodogen method as previously reported (3). Monoiodinated GnRHA was separated from unreacted iodine, GnRHA, and diiodinated GnRHA by chromatography over QAE-Sephadex (Sigma) that had been equilibrated with 50 mM ammonium bicarbonate (pH 9.7) and packed into a 10-ml plastic pipette. The fractions of peak activity (usually between 13–17 ml from the column) were pooled, lyophilized to dryness, rehydrated with 2 ml deionized water, relyophilized twice, and finally stored under nitrogen gas at −20 C overnight before azido-derivatization (15). The [^{125}I]GnRHA plus any bicarbonate residue was dissolved in 400 μl methanol-triethylamine (10 ml:35 μl; pH to 8.0 with HCl). Eight milligrams of hydroxysuccinimide azidobenzoate (Pierce Chemical Co., Rockford, IL) was dissolved in 60 μl dimethylformamide (Pierce Chemical Co.) and added to the solubilized [^{125}I]GnRHA. The reaction was placed in the dark for 2–5 h and then fractionated over a C_{18} reverse phase column (300 × 3.9 mm; Phenomenex, Torrance, CA) that was equilibrated with 20% acetonitrile-80% deionized water containing 0.1% trifluoroacetic acid (Pierce Chemical Co.) at a flow rate of 1 ml/min. Twelve fractions were collected before the initiation of step changes to 30%, 40%, 50%, 60%, and 70% acetonitrile; four 1-ml fractions were collected from each step change. The [^{125}I]azidobenzoyl-GnRHA (referred to as [^{125}I]azido-GnRHA throughout the text) eluted routinely in 1 ml at fraction 22. The active fraction(s) was collected in a siliconized plastic tube and lyophilized for 0.5 h on a rotary vacuum (Savant Instruments, Inc., Hicksville, NY) to remove acetonitrile and was stored at −20 C in the dark for up to a month. Specific activities were routinely 1300–1900 μCi/μg, and consistent results were obtained with fresh derivative (<1 week old).

Photoaffinity labeling of GnRHRec

Cells were thawed and resuspended in 2 ml filter-sterilized assay buffer/million cells [10 mM Tris-Cl (pH 7.4) containing 0.1% BSA (Sigma) and 1% brain-heart infusion (Becton Dickinson and Co., Cockeysville, MD) to minimize nonspecific background labeling]. Cells were centrifuged at 2000 rpm for 5 min at 4 C, resuspended in assay buffer at a concentration of 3 million cells/0.4 ml, and then distributed to 50-ml conical plastic culture tubes (3 million cells/tube unless indicated otherwise). Freshly prepared [^{125}I]azido-GnRHA (100,000–200,000 cpm) was added to the cell suspensions in low laboratory light, and the mixture was incubated together for 4 h with tubes gently rotating in the dark at a 45° angle at 4 C. When unlabeled GnRH was added to compete for specific receptor binding, it was added along with [^{125}I]azido-GnRHA during the 4-h binding reaction. After binding, cells were diluted with 3 ml ice-cold wash buffer (10 mM Tris-Cl, pH 7.4), immediately poured into a 15-ml siliconized glass tubes, placed into a 4 C water bath at a distance of 3 in. from a 450-watt mercury vapor lamp (Ace-Hanovia Photochemical Lamp, Ace Glass, Inc., Vineland, NJ) (25), and exposed to the high intensity light for 6 min. The mixture was poured into a 5-ml polypropylene culture tube and centrifuged at 30,000 × *g* for 10 min to pellet the photoaffinity radiolabeled cells. Samples were usually fractionated by SDS-PAGE immediately, but were sometimes stored at −80 C for several days without breakdown of the radiolabeled complex.

Denaturing PAGE

Samples were resuspended in 100 μl SDS-PAGE sample buffer [15 mM Tris-Cl (pH 6.8), 0.5% SDS, 10% glycerol, 5% β-mercaptoethanol, and bromophenol blue]. Each sample was sonicated twice for 10 sec each time with a 1/8th-in. tipped probe (model LS75, level 7, Branson Ultrasonic Corp., Stanford, CT) and then incubated at 37 C for 1 h in sealed tubes. Twenty to 80 μl of each sample were applied to a 1.5-mm thick SDS-polyacrylamide gel in a Hoefer electrophoretic apparatus (model SE600, Hoefer Scientific, San Francisco, CA) with 4.5% stacking gel and 10% running gel. Electrophoresis through the stacking gel was performed at 15 mA followed by electrophoresis at 30 mA until the dye front reached the bottom of the gel. Gels were fixed and stained in 0.5% Coomassie blue for 30 min and then destained overnight, vacuum dried at 60 C, and placed on a phosphorimager cassette for 24–48 h. Results were visualized using a 445S PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) and quantitated using Molecular Dynamics, Inc. ImageQuant software.

Treatment with calf intestinal phosphatase (CIP)

One million cells were freshly photoaffinity labeled with [^{125}I]azido-GnRHA, resuspended in 100 μl SDS-PAGE sample buffer without bromophenol blue dye, and incubated at 37 C for 1 h to solubilize the GnRHRec. To prevent denaturation of CIP (or *N*-glycosidase F; see below) the SDS concentration was lowered to 0.01% by diluting the 100-μl samples with 1.9 ml PBS and then concentrating them in Centricon-30 filter units (Amicon, Danvers, MA); this procedure was performed twice. To prevent proteolysis in the absence of SDS during enzymatic incubation, the following protease inhibitors were included in the PBS wash solution noted above [pepstatin (Sigma) at 1 μg/ml, leupeptin (Sigma) at 1 μg/ml, and 1 mM Pefabloc-SC (Roche Molecular Biochemicals, Indianapolis, IN)]. Protein extracts were transferred to 1.5-ml microfuge tubes and adjusted to 90 μl with PBS containing protease inhibitors without the bromophenol blue dye. Ten microliters of 10 × CIP buffer [500 mM Tris-Cl (pH 8.5) and 1 mM EDTA] and 20 U CIP (BRL, Bethesda, MD) were added in 10 μl and incubated for 8 or 24 h at 37 C. As a positive control, 1 μg phospho-casein was treated in the same manner. After incubation, the enzymatically treated proteins were fractionated by SDS-PAGE.

Western blot analysis of phosphoserine on phospho-casein

After incubation with or without CIP, the casein samples were resolved by SDS-PAGE as noted above. The gel was sliced into two halves, and one portion was stained with Coomassie blue stain. Proteins on the other half were transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell, Inc., Keene, NH). The membrane was blocked with 10 mM Tris (pH 7.5) and 30 mM NaCl containing 0.1% Tween-20 and 5% BSA and probed with antiphosphoserine antibody (Sigma) at a 1:1000 dilution. Immunoreactive proteins were detected using enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech, Arlington Heights, IL).

Deglycosylation

Treatment with *N*-glycosidase F (Oxford GlycoSciences, Boston, MA) was similar to CIP treatment (see above), except that the protein extracts were transferred to 1.5-ml microfuge tubes and adjusted to 80 μl (instead of 90 μl) with PBS containing protease inhibitor buffer. Twenty microliters of glycosidase 5 × buffer were added [20 mM sodium phosphate (pH 7.5) and 50 mM EDTA] followed by 5 U *N*-glycosidase F, and samples were incubated at 37 C for 8 or 24 h. After incubation, the labeled GnRHRec in each sample was analyzed by SDS-PAGE. As a positive control, GnRHRec from αT3-1 cells were photoaffinity labeled with [^{125}I]GnRHA, treated in the same manner with *N*-glycosidase F, and analyzed by SDS-PAGE.

Treatment with tunicamycin

Ovine pituitary cells were cultured in six-well multiwell plates and treated with 10 nM estradiol plus 2 μg/ml tunicamycin [Sigma; 200 μg/ml dissolved in 1 ml ethanol-water (2:1) stock solution] for 6, 12, 24, 48, and 72 h. Cells were harvested, and GnRHRec was labeled with [^{125}I]azido-GnRHA before analysis by SDS-PAGE.

Statistical analysis of data

All studies were performed at least three times with similar results. The quantitative data in Fig. 4 represent the mean and SEM from four ovine pituitary cultures as described in the legend. Figures 2 and 5–7 show representative data that are qualitatively similar to results obtained with three to seven independent pituitary preparations.

Results

Estradiol-mediated inhibition of GnRH-stimulated LH secretion

The data in Fig. 1 show that GnRH-stimulated LH release was inhibited after 6 h of treatment with 10 nM estradiol; in this case, LH release was decreased to basal levels by 72 h.

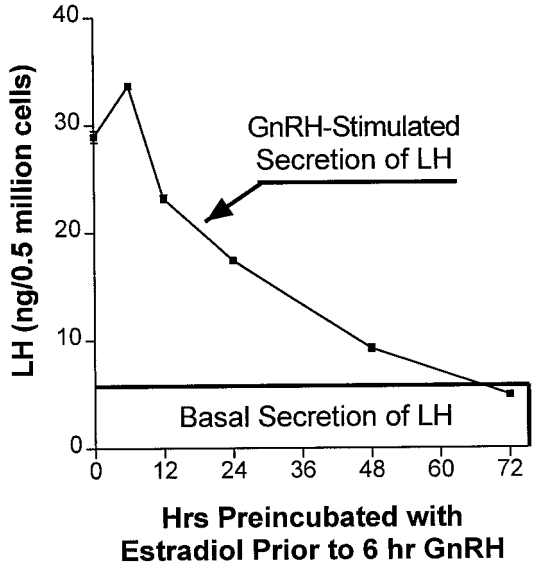

FIG. 1. Inhibition of GnRH-stimulated LH secretion by more than 6 h of treatment with estradiol. Cultures were pretreated with 10 nM estradiol for 0, 6, 12, 24, 48, and 72 h; the media were changed, and cultures were treated with 10 nM GnRH for 6 h more in the presence of estradiol. All cultures were treated with GnRH at the same time, so the age of the culture was not a factor in GnRH secretion (preincubations with estradiol were staggered before GnRH addition). The data represent the mean ± SEM of three replicates from one of the ovine pituitary cultures used for data in Fig. 4, A and B. In most cases the SEM is within the data symbol.

Previous reports of estrogen acton (3, 14) have emphasized the initial increase in GnRH-stimulated LH secretion that is usually observed (2- to 3.5-fold increase), but which is seen here only briefly (at 6 h) (3, 14). Apparently, GnRH-stimulated LH secretion was near maximal levels in this particular culture before estrogen treatment, which permitted the observation of almost pure negative regulation of the GnRH response by estradiol.

Photoaffinity labeling of the ovine GnRH receptor

The data shown in Fig. 2 demonstrate that the ovine GnRHRec was specifically photolabeled with [^{125}I]azido-GnRHA and migrated with an apparent M_r near 38 kDa. Induction of the GnRHRec by estradiol and/or inhibin was expected from many previous experiments (3–5, 13, 14, 17), and estradiol induced what appeared to be two GnRHRec species: 38 kDa and a larger type near 43 kDa. Inhibin induced only the 38-kDa GnRHRec. The induced GnRHRec bands were competed away with 100 nM GnRH (Sigma), but two nonspecific bands at 57 and 23 kDa were not competed away with GnRH. Other nonspecific bands were occasionally observed, as shown in Figs. 4 and 7.

It should be noticed that background counts near 38–48 kDa in Fig. 2 (also see Figs. 4–7) were extremely low, making analysis of radiolabeled GnRHRec more sensitive and accurate than traditional binding assays (3–5). Even so, the image in Fig. 2 had to be overexposed to show the faint band of GnRHRec in lane 1; this band was always difficult to detect in cultures not treated with either estradiol or inhibin (see Figs. 4 and 6). The low control levels of GnRHRec may reflect inhibition of GnRHRec expression by activin (26) in static

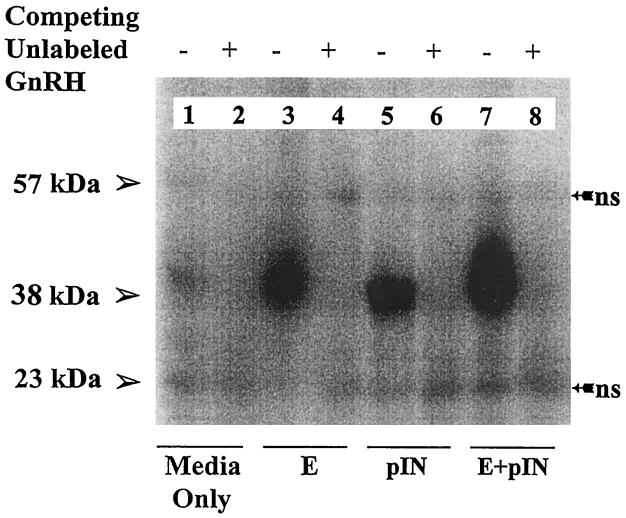

FIG. 2. Photoaffinity labeling of ovine GnRHRec with [^{125}I]azido-GnRHA. Cells were treated for 36 h with culture medium alone (lanes 1 and 2) or with medium containing 10 nM estradiol (E; lanes 3 and 4), 10 μg/ml porcine follicular inhibin (pIN; lanes 5 and 6), or both E and pIN (lanes 7 and 8); scraped from culture dishes; and photoaffinity labeled with [^{125}I]azido-GnRHA (see *Materials and Methods*). Samples were incubated without (−) or with (+) 100 nM GnRH designed to block specific labeling of the GnRHRec (see lanes 2, 4, 6, and 8). ns, Nonspecific labeling.

ovine cultures, as addition of follistatin to these cultures [(known to block activin action (27)], induces GnRHRec identically to inhibin (Miller, W. L., unpublished data). Because of the extremely low levels of control GnRHRec, neither estradiol nor inhibin induction is reported in terms of fold induction, because this measure varied dramatically from 10- to more than 300,000-fold based on the variable ability to detect control levels of GnRHRec.

Establishing a single point assay for measuring GnRHRec

Previous studies using traditional single point binding assays showed that maximal binding of GnRH to its receptor was obtained when ovine pituitary cultures were pretreated with estradiol plus an enriched preparation of porcine inhibin (4); the lowest level of binding occurred after progesterone treatment (17). As none of the previous binding studies from our laboratory was calibrated for linearity of response, an effort was made here to establish parameters that would permit accurate quantitation using a single point assay. The conditions used in Fig. 3 were chosen from several dose-response studies reported previously (28) establishing optimal conditions for binding.

The data in Fig. 3 demonstrate the linearity of response obtained when increasing levels of GnRHRec were assayed, whereas the total number of cells and [^{125}I]azido-GnRHA were kept constant. The response from beginning to end was linear, with an r value of 0.988. The amount of radiolabeled GnRHA covalently bound to cells containing the highest level of GnRHRec (E/pIN-treated cells) was 8 ± 0.4 fmol/10 million cells. This represents 23 ± 1% of the theoretical maximum level of GnRHRec in these cells calculated by Scatchard analysis (3, 17). The parameters used in Fig. 3 were used in all figures presented below.

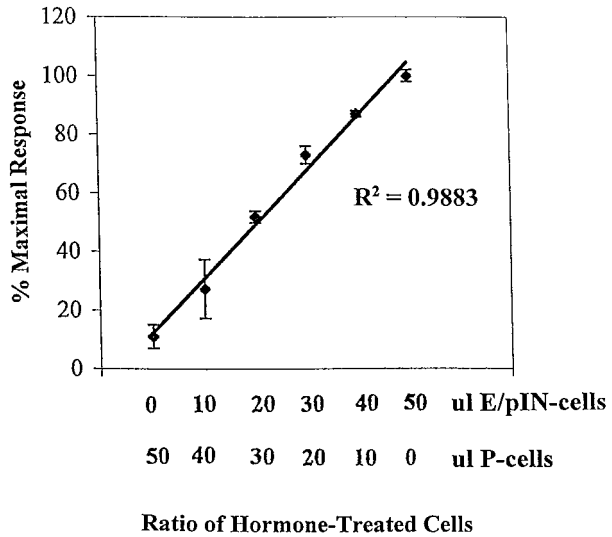

FIG. 3. Linear dose-response of GnRHRec photoaffinity labeling. Cells were incubated with estradiol (E) and porcine inhibin (pIN; 48 h) to induce maximal levels of GnRHRec or with 100 nM progesterone (P) for 48 h to reduce GnRHRec to its lowest level possible. These E/pIN- and P-treated cells were scraped, washed, resuspended in binding buffer at 3 million cells/50 μl, and mixed in E/pIN:P proportions of 0:50, 10:40, 20:30, 30:20, 40:10, and 50:0. These cell mixtures were then incubated with 100,000 cpm [^{125}I]azido-GnRHA in a total of 0.4 ml binding buffer, photoaffinity labeled, and analyzed as described in Fig. 2. The 38–43 kDa region was quantitated and plotted as a percentage of the maximal response obtained with 50 μl E/pIN-treated cultures. Data represent the mean ± SEM of three separate experiments, each performed in triplicate.

Induction of GnRHRec by estradiol and/or recombinant human inhibin A

Figure 4A shows data from a typical time-course study in which cells were treated with 10 nM estradiol for 0, 6, 12, 24, 48, or 72 h. There was a shift in size of the GnRHRec after 24 h of estradiol treatment. This shift occurred after the LH response to GnRH had already been diminished (by 24 h) (3). The data in Fig. 4, A, C, and E, are from a single, but representative, preparation of ovine pituitary cells in culture; the estradiol-induced shift in GnRHRec size after 24 h (Fig. 4A) was observed in all pituitary preparations studied (see also Figs. 2, 4C, and 5–7). Note that phosphorimager band intensities were adjusted to accurately show the relative positions of the bands; because intensities were adjusted, they do not always appear to correlate exactly with the quantitative data shown in the bar graphs. Figure 4B shows a bar graph depicting phosphorimager quantitation of the GnRHRec. The bar graph included data from Fig. 4A plus data from three other independent pituitary preparations (see figure legend). Induction of GnRHRec by estradiol occurred rapidly (always ≥50% complete within 6 h) and remained high until 60 h, after which it decreased somewhat.

The data in Fig. 4, C and E, were obtained from cultures treated with either 10 nM estradiol plus 10 ng/ml recombinant inhibin A (Fig. 4C) or 10 ng/ml recombinant inhibin A alone (Fig. 4E). In either case, a single 38-kDa GnRHRec was induced at 6, 12, and 24 h in the presence of inhibin alone or estradiol plus inhibin (Fig. 4C). The size of GnRHRec in-

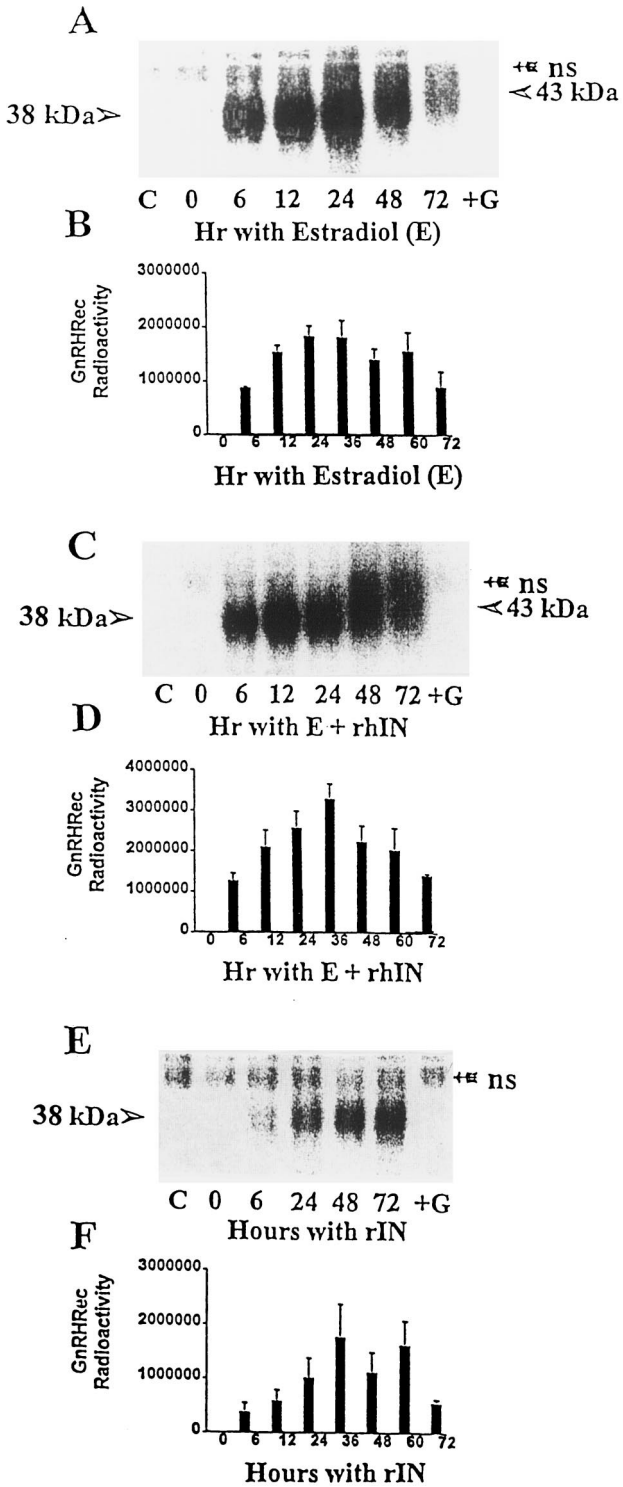

FIG. 4. Kinetics of induction and hyperglycosylation of GnRHRec by estradiol and inhibin. Cell cultures were treated with 10 nM estradiol (A and B), 10 ng/ml recombinant inhibin (E and F), or both (C and D) for 0, 6, 12, 24, 36, 48, 60, and 70 h. Cells were labeled with [^{125}I]azido-GnRHA and analyzed as described in Figs. 2 and 3. The qualitative data in A, C, and E are representative of results from more than 12 independent preparations. Band intensities have been adjusted to accurately show the relative positions of the bands; because intensities have been adjusted, they do not always correlate well with the quantitative data shown in the bar graphs. The quantitative data (bar graphs) were obtained from 4 independent time-course studies. Two studies included points at 0, 6, 12, 24, 36, 48, and 60 h, whereas the other 2 included points at 0, 6, 12, 24, 48, and 72 h (only the rIN treatment in E lacked the 12 h point). As culture to culture variation existed, all quantitative data from the 3 treatments in the 4 preparations were multiplied by a factor that adjusted the value at the 24 h point for estrogen treatment only to the average value obtained for that particular treatment from all 4 preparations (1,800,000; Fig. 3B at 24 h). The quantitative data represent the mean ± SEM of 4 single values from the 4 independent culture preparations, except for the 36, 60, and 72 h points (only 2 values were available at these times, so ranges were plotted). Lanes designated C and G contained photoaffinity-labeled material from cells incubated in the presence of 100 nM unlabeled GnRH to eliminate specific binding of [^{125}I]azido-GnRHA. C represents the lowest value possible in the 38–43 kDa range (used as zero for quantitation), and G verifies that the induced bands between 38–43 kDa were, in fact, GnRHRec. ns, Nonspecific binding.

creased to 43 kDa between 24–72 h only when estradiol was present (Fig. 4, C *vs.* E).

Quantitation of induction by inhibin alone (Fig. 4F) indicated that inhibin increased GnRHRec more slowly and to a lesser extent than estradiol (Fig. 4B); it took 24 h for inhibin to reach an average radioactivity level of 1.2 million (maximal radioactivity), whereas estradiol nearly reached that level within 6 h and reached 1.8 million (maximal radioactivity) by 24 h. Data not shown here, but relevant to the low level of GnRHRec in static cultures (noted above in Fig. 2), indicate that treatment with follistatin (250 ng/ml) causes the same induction as inhibin. The results for inhibin and follistatin treatment were identical. The results comparing follistatin and inhibin were obtained from four different pituitary preparations.

Induction of GnRHRec by estradiol plus inhibin was essentially the sum of induction caused by the individual treatments. This was true when comparing time point to time point, except for the 60 h point (note the difference in scales for Fig. 4B/4F *vs.* Fig. 4D). The mean ± SEM of total radioactivity for four separate experiments in the presence of estradiol plus inhibin was 7.97 ± 1.2 million (summed over the points of 6, 12, 24, and 48 h shown in Fig. 4D). The equivalent radioactivity summed over the same time points for estradiol alone added to inhibin alone was 8.65 ± 1.1 million. These were not statistically different values, indicating that the effects of estradiol and inhibin were additive.

Phosphatase treatment of the GnRHRec

To study whether phosphorylation played a role in the observed size shift of GnRHRec, the GnRHRec was subjected to phosphatase treatment. To make certain that CIP was active under the conditions used for GnRHRec dephosphorylation, a known phosphoprotein (phospho-casein) was processed exactly as GnRHRec and then exposed to CIP.

The data presented in Fig. 5A (*two left lanes*) show that phosphatase treatment of phospho-casein significantly changed the mobility of the upper band (77 kDa) so that it migrated as if it were 70 kDa. Figure 5A (*two right lanes*) shows that phosphatase treatment completely dephosphorylated the phospho-casein, as the phosphoserine signal disappeared in the phosphatase-treated lane (+ CIP). The data in Fig. 5B indicate that phosphatase treatment had no effect on the mobility of GnRHRec produced in αT3-1 cells that migrated between 47–54 kDa. The estradiol-induced, large

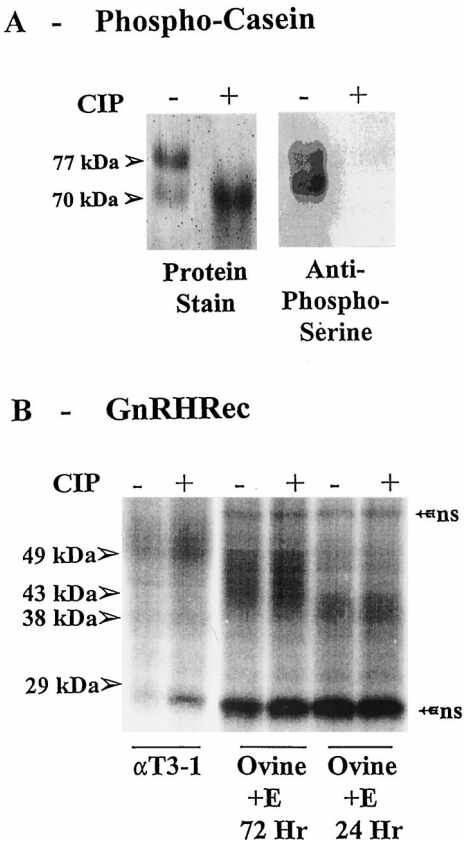

FIG. 5. Treatment of phosphocasein and GnRHRec with CIP. A (*Two left lanes*), One-microgram samples of phosphocasein were incubated for 8 h at 37 C with (+) or without (−) 20 U CIP. After digestion, the samples were analyzed by SDS-PAGE followed by protein visualization with Coomassie blue as described in *Materials and Methods*. A, *Two right lanes*, Same as the *left two lanes* except that phosphocasein was transferred to nitrocellulose membranes after SDS-PAGE and probed with antiphosphoserine antibodies (see *Materials and Methods*). B, Ovine pituitary cells were treated for either 24 or 72 h with 10 nM estradiol. Also, αT3-1 cells were harvested, and samples of one million cells each from these three different cell populations were photoaffinity labeled with [^{125}I]azido-GnRHA, solubilized in the standard fashion before electrophoresis, and incubated with (+) or without (−) CIP for 8 h at 37 C after SDS was decreased in the samples (see *Materials and Methods*). After enzymatic digestion, the samples were analyzed by SDS-PAGE and phosphorimager. ns, Nonspecific binding. These results are similar to those obtained in three identical, but independent, experiments.

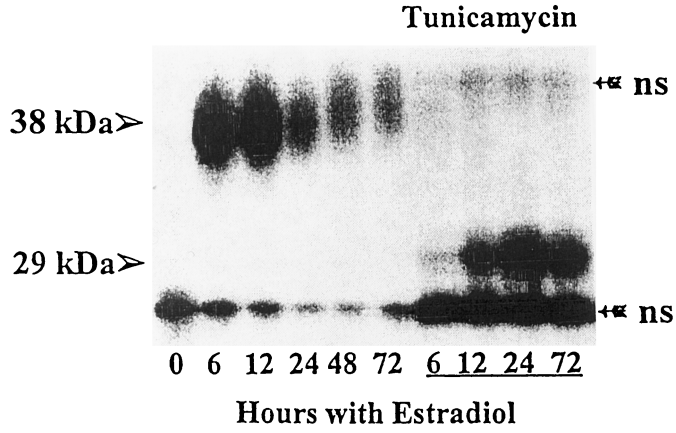

FIG. 6. Induction of a nonglycosylated ovine GnRHRec. Ovine pituitary cells were incubated with 10 nM estradiol for 0, 6, 12, 24, 48, and 72 h as described in Fig. 4A, and the phosphorimager scan is shown on the *left*. Cells from the same culture preparation were treated with estradiol along with tunicamycin (see *Materials and Methods*) for 6, 12, 24, and 72 h. Induction of a 29-kDa GnRHRec species occurred, and there was no shift in apparent M_r between 24 and 72 h. These results are representative of three identical, but independent, experiments.

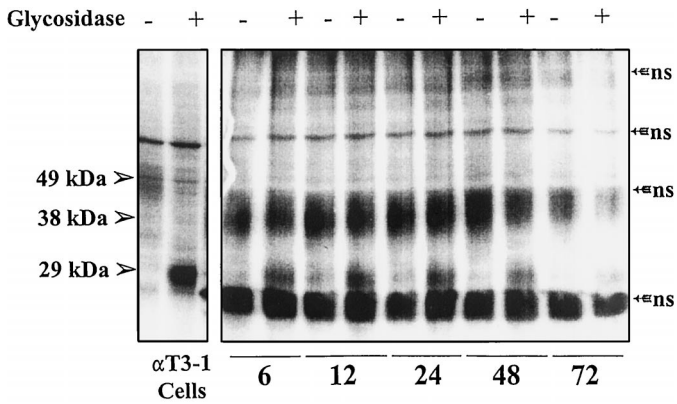

FIG. 7. Deglycosylation of the GnRHRec with *N*-glycosidase F. Ovine pituitary cultures were treated with estradiol for 6, 12, 24, 48, and 72 h. Then they, along with αT3-1 cells, were photoaffinity labeled with [^{125}I]azido-GnRHA as described in Fig. 5. The cells were processed to remove most of the SDS as described in Fig. 5, treated for 8 h with (+) or withouth (−) *N*-glycosidase F, and then analyzed by SDS-PAGE and phosphorimaging. The data are representative of three similar, but independent, experiments.

(43-kDa) GnRHRec at 72 h appeared to be unaffected by CIP, and there was no effect of CIP on the 38-kDa GnRHRec (estradiol treatment for 24 h). These data do not prove that phosphates were not removed from GnRHRec during the procedure, but they clearly show that if dephosphorylation occurred, it failed to change the mobilities of either 38- or 43-kDa GnRHRec.

Estrogen induction of nonglycosylated GnRHRec in the presence of tunicamycin

As the shift in M_r of GnRHRec did not appear connected with phosphorylation (Fig. 6), the possibility of glycosylation was investigated. The complementary DNA (cDNA) for ovine GnRHRec predicts a M_r of 37.6 kDa (29), so the 38-kDa GnRHRec was thought to be nonglycosylated, and the shift to 43 kDa might represent addition of carbohydrate. Figure 6 shows data from a study in which GnRHRec was induced by estradiol in the absence or presence of tunicamycin, which prevents glycosylation of proteins. The data in the *six left lanes* of Fig. 6 show induction of GnRHRec like that in Fig. 4A where a 38-kDa GnRHRec was first induced and then shifted to a 43 kDa size between 24–48 h. The *four right lanes* (with tunicamycin) showed significant induction of the ovine GnRHRec in the presence of tunicamycin, but instead of a 38-kDa GnRHRec being formed at all time points, an unexpected 29-kDa GnRHRec appeared. The 29-kDa GnRHRec did not increase in size after 24 h, indicating that the estradiol-mediated size shift probably involves glycosylation,

which was prevented by tunicamycin. It is worth noting that the estrogen-mediated decrease in the LH response to GnRH still occurred even in the presence of tunicamycin (as measured by GnRH-stimulated LH secretion; data not shown), so the observed decrease in the LH response to GnRH appeared to be uncoupled from glycosylation and, thus, the change in size.

Deglycosylation of the GnRHRec

As tunicamycin treatment resulted in a lower than expected, 29-kDa form of GnRHRec, it was decided to confirm the results by enzymatically deglycosylating normal GnRHRec (38 kDa) and the putative hyperglycosylated GnRHRec (43 kDa). The data in Fig. 7 indicate that removal of carbohydrate from the GnRHRec created a deglycosylated receptor that migrated with an apparent M_r of 29 kDa, identical to that in the tunicamycin study. This nonglycosylated band was narrower and less diffuse than the 38- or 43-kDa bands. As a positive control, the first two lanes show αT3-1 GnRHRec before and after deglycosylation. Nearly all of the αT3-1 GnRHRec was deglycosylated to form a 29-kDa receptor, although its cDNA predicts a M_r of 37.6 kDa also (2). The remaining lanes show deglycosylation of ovine GnRHRec from cultures treated with estradiol for 6, 12, 24, 48, and 72 h. In the absence of *N*-glycosidase F, the GnRHRec showed the typical increase in M_r from 38 to 43 kDa after 24 h of estradiol treatment. Treatment with *N*-glycosidase F uniformly generated a 29-kDa GnRHRec, although deglycosylation was only partially complete during the 8-h incubation. It is not known why deglycosylation of the ovine GnRHRec was so inefficient and not complete as that with the αT3-1 GnRHRec; a longer, 24-h incubation (not shown) caused general degradation of the ovine GnRHRec. The major point, however, is that a 29-kDa ovine GnRHRec species was formed from either 38- or 43-kDa GnRHRec in all samples. These data indicate that the estradiol-induced shift in M_r from 38 to 43 kDa was created by the addition of carbohydrate to the 38-kDa GnRHRec form to create a 43-kDa family member.

Discussion

GnRHRec has been identified in many tissues of the body through its ability to specifically bind [^{125}I]GnRHA [specific nuclei in the central nervous system (30), ovary (31, 32), testis (32), placenta (33), various tumors of the pituitary and pancreas (34), and pituitary gonadotropes and somatogonadotropes (35, 36)]. In most of these vertebrate tissues the GnRHRec seems to be involved with regulation of reproductive tissue. At the pituitary, alone, its effects are pleiotropic and quite diverse; it causes 1) transcriptional induction of the α, LHβ, and FSHβ genes; 2) secretion of LH and FSH; and 3) regulation of its own mRNA level. To date, however, only few molecular details of these actions are well understood.

It has been known for some time that estradiol and inhibin increase GnRH-stimulated LH release in ovine pituitary cultures, but that extended exposure of pituitary cells to estradiol (>6 h) has a progressive negative impact on GnRH-stimulated LH secretion, as observed in Fig. 1. Our studies were intended to determine whether this inhibition was due to modification of the GnRHRec as observed in preliminary studies (16). The effects of estradiol and inhibin on GnRHRec synthesis have been analyzed here in more detail than ever before.

A number of studies have shown that both estradiol and inhibin can increase the amount of [^{125}I]GnRH binding in static cultures of ovine pituitaries (3–5, 17, 26). Previous studies from our laboratory have shown complete additivity of the effects of estradiol and inhibin induction at the steady state mRNA level (4, 13), but single point GnRH binding assays showed only partial additivity at the GnRHRec protein level (4). It was important to determine whether nonadditivity of induction at the protein level was truly occurring or if estradiol and inhibin interacted in a way that prevented full additivity at the GnRHRec level. The data presented here show unequivocally that changes at the protein level do, in fact, mirror the complete additivity of regulation observed at the mRNA level. Thus, the effects of inhibin or estradiol influence the pituitary system in totally independent ways, with each carrying out its designated role without interference from the other.

Because levels of GnRHRec were so low in the untreated static cultures reported here, this is likely to be an artifact of static pituitary cultures, which, fortuitously, permitted the clear analyses of inhibin and/or estradiol actions. Recently, our laboratory has shown that an activin-like molecule that is incapacitated by follistatin (possibly bone morphogenic protein-6/7) rapidly accumulates in pituitary cultures to stimulate FSH production (37). Postulating that this factor might also inhibit GnRHRec synthesis, follistatin was added to cultures to inactivate the substance and thereby increase GnRHRec which it did as well as inhibin (data not shown, but they were identical to inhibin data in Fig. 4). Our conclusion, therefore, is that static ovine pituitary cultures produce a substance that stimulates FSH synthesis, blocks GnRHRec synthesis, and is counteracted by follistatin and inhibin. As build-up of this material is likely to vary from one pituitary culture to another and is surely lower *in vivo,* where blood flow washes away paracrine and autocrine factors, it will be difficult to determine the physiological importance of this factor until it is immunoneutralized in sheep to determine its effect on GnRHRec *in vivo*.

The primary reason for performing the studies reported here was to learn more about the nature of the GnRHRec itself and to discover, in particular, how estradiol can alter the GnRHRec to increase its apparent M_r and perhaps control its ability to respond to GnRH stimulation. Iwashita and Catt first visualized a single GnRHRec migrating near 38 kDa on isolated plasma membranes from ovine pituitaries (39 $\pm$ 0.5 kDa) (15). They analyzed tissues obtained from slaughterhouse animals of unknown sex or reproductive status, however, so it was unclear whether reproductive cycle hormones would or could modify the observed GnRHRec. The studies reported here indicate that a higher M_r form of GnRHRec (43 kDa) can be induced by prolonged estrogen treatment such as that occurring before and during the preovulatory LH surge *in vivo*. Based on the studies presented here, the increase in apparent M_r of the ovine GnRHRec is caused by estradiol-induced hyperglycosylation of the 38-kDa

GnRHRec. By contrast, inhibin induced only the 38-kDa M_r species of GnRHRec.

It was of interest to characterize the time course of estradiol-mediated hyperglycosylation and to compare it to the kinetics of estradiol-mediated desensitization of the LH response to GnRH. It was determined that hyperglycosylation occurs sometime after 24 h of estradiol treatment, but that estrogen-induced desensitization of the LH response begins somewhere between 12–15 h, at a time when increased glycosylation had not yet begun. Therefore, it was concluded that changes in glycosylation are unlikely to cause the desensitization. This conclusion is strongly supported by the fact that desensitization can be observed (data not shown) even in the presence of tunicamycin when glycosylation is fully inhibited.

The effect of estradiol to glycosylate proteins may be general rather than specific for the GnRHRec in pituitary gonadotropes, a fact that makes it difficult to determine the physiological function of hyperglycosylated GnRHRec. In this regard, it is interesting to note that estradiol increases the carbohydrate content of LH after only 4 h in rat pituitary cultures (38). Apparently estradiol increases the ability of gonadotropes to add carbohydrate to their secretory proteins (*e.g.* LH and FSH) and membrane-bound molecules (GnRHRec) simultaneously. Although increased glycosylation does not appear to alter the affinity of GnRHRec for GnRH (3), it may increase GnRHRec stability or efficiency of transport to the membrane (21).

A surprising observation of this study was the finding that normal 38-kDa ovine GnRHRec is highly glycosylated, presumably on asparagine 18. Based on the 37.6 kDa M_r calculated for the ovine (29) and murine (2) GnRHRec proteins from their cDNA sequences, the 38-kDa receptor would not be expected to contain carbohydrate. However, induction of ovine GnRHRec in the presence of tunicamycin yielded a much lower M_r GnRHRec species of 29 kDa, which is 9 kDa less (24% less) than predicted (see Fig. 6). Furthermore, deglycosylating either the 38- or 43-kDa ovine GnRHRec yielded the same 29-kDa M_r GnRHRec, and this occurred also with the murine GnRHRec, which is known to be heavily glycosylated.

It is not clear why nonglycosylated GnRHRec migrates at such an anomalous position on SDS-PAGE. All G protein-coupled receptors contain seven transmembrane regions that are quite hydrophobic, and they might be expected to bind more SDS than normal proteins, which could cause them to migrate faster than normal on SDS-PAGE, but most G protein-coupled receptors do, in fact, migrate on SDS-PAGE according to the M_r predicted from their cDNAs. It may be that the GnRHRec has a unique structure that keeps it from unfolding completely during the 1-h treatment with SDS-β-mercaptoethanol at 37 C. Alternatively, it could be postulated that the GnRHRec is modified after translation to become a truncated receptor, but data from the laboratory of Dr. T Nett (39) suggest that this is not the case. Dr. Nett's laboratory transcribed full-length mouse GnRHRec mRNA from a well characterized GnRHRec cDNA *in vitro* and then translated it *in vitro* to produce a molecule that migrated between 25–30 kDa on SDS-PAGE. Presumably, the *in vitro* transcription-translation systems had no enzymes to modify either the mouse GnRHRec mRNA or protein, so the 25- to 30-kDa protein should have been full-length mouse GnRHRec without any modification(s). Thus, the reason for the anomalous migration of the nonglycosylated GnRHRec on SDS-PAGE remains unknown, but the phenomenon does exist.

Finally, one goal of this paper was to determine whether phosphorylation of the GnRHRec was involved in the estradiol-induced increase in GnRHRec size. The conclusion here is that only glycosylation has been associated with the shift in GnRHRec electrophoretic mobility. It is still possible that estradiol decreases gonadotrope responsiveness to GnRH by phosphorylating the GnRHRec, but definitive analysis of GnRHRec phosphorylation awaits future advances in gonadotrope study.

In summary, the ovine gene for GnRHRec encodes a protein that has a calculated M_r of 37.6 kDa (based on its cDNA sequence), but in its nonglycosylated form, it migrates as if it were a 29-kDa protein on SDS-PAGE. Glycosylation of the ovine GnRHRec, presumably on asparagine 18, causes it to migrate as a 38-kDa glycoprotein on SDS-PAGE (the native ovine GnRHRec), and the receptor can be further glycosylated to migrate at 43 kDa after more than 24 h of treatment with 10 nM estradiol. Although it is known that estradiol treatment can decrease the ability of pituitary cells to respond to GnRH even as it induces higher levels of GnRHRec, the data reported here indicate that the decrease in GnRH effectiveness does not appear to be associated with hyperglycosylation of the ovine GnRHRec, as desensitization to GnRH occurs before hyperglycosylation begins. Finally, it has been known that both inhibin and estradiol induce the production of GnRHRec in ovine pituitary cells in culture; the data presented here show that the effects of estradiol and inhibin are additive at the GnRHRec protein level.

Acknowledgments

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