Lamprey GnRH-III Acts on Its Putative Receptor via Nitric Oxide to Release Follicle-Stimulating Hormone Specifically[1]

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Lamprey gonadotropin-releasing hormone-III (l-GnRH-III), the putative follicle-stimulating hormone (FSH)-releasing factor (FSHRF), exerts a preferential FSH-releasing activity in rats both *in vitro* and *in vivo*. To test the hypothesis that l-GnRH-III acts on its own receptors to stimulate gonadotropin release, the functional activity of this peptide at mammalian (m) leutinizing hormone (LH)RH receptors transfected to COS cells was tested. l-GnRH-III activated m-LHRH receptors only at a minimal effective concentration (MEC) of 10^{-6} M, whereas m-LHRH was active at a MEC of 10^{-9} M, at least 1,000 times less than that required for l-GnRH-III. In 4-day monolayer cultured cells, l-GnRH-III was similarly extremely weak in releasing either LH or FSH, and, in fact, it released LH at a lower concentration (10^{-7} M) than that required for FSH release (10^{-6} M). In this assay, m-LHRH released both FSH and LH significantly at the lowest concentration tested (10^{-10} M). On the other hand, l-GnRH-III had a high potency to selectively release FSH and not LH from hemipituitaries of male rats. The results suggest that the cultured cells were devoid of FSHRF receptors, thereby resulting in a pattern of FSH and LH release caused by the LHRH receptor. On the other hand, the putative FSH-releasing factor receptor accounts for the selective FSH release by l-GnRH-III when tested on hemipituitaries. Removal of calcium from the medium plus the addition of EGTA, a calcium chelator, suppressed the release of gonadotropins induced by either l-GnRH-III or LHRH, indicating that calcium is required for the action of either peptide. Previous results showed that sodium nitroprusside, a releaser of nitric oxide (NO), causes the release of both FSH and LH from hemipituitaries incubated *in vitro*. In the present experiments, a competitive inhibitor of NO synthase, L-*N*G-monomethyl-L-arginine (300 μM) blocked the action of l-GnRH-III or partially purified FSHRF. The results indicate that l-GnRH-III and FSHRF act on putative FSHRF receptors by a calcium-dependent NO pathway. Exp Biol Med 227:786–793, 2002

Key Words: m-LHRH receptors; FSHRF receptors; NO synthase; cGMP.

Previous studies indicated that there is a separate hypothalamic control of follicle-stimulating hormone (FSH) release distinct from that of luteinizing hormone (LH) (1–3). An FSH-releasing factor (RF) was purified from sheep (4–6) and rat hypothalami (6). In recent studies, we reported that lamprey (l) gonadotropin-releasing hormone III (GnRH-III) has a preferential FSH-releasing action *in vitro* and *in vivo* (7). Gel filtration on Sephadex G-25 of sheep stalk median eminence fragments or rat hypothalami has repeatedly separated a fraction with selective FSH-releasing activity both *in vivo* and *in vitro*. In a recent experiment, the FSH-releasing fraction contained l-GnRH as determined by radioimmunoassay (RIA) and its activity was neutralized by an antiserum against l-GnRH, suggesting that l-GnRH-III or a closely related analog is FSHRF (6).

Lamprey GnRH-III is a decapeptide that has 60% homology with m-LHRH with different amino acids in positions 5–8 (8). It was originally reported that it was involved in reproduction based on its ability to stimulate steroidogenesis and gametogenesis in adult sea lampreys and of the occurrence of this peptide in different stages of metamorphosis coinciding with the acceleration of gonadal maturation in lampreys (8, 9).

Both l-GnRH I and III neurons are localized in the lamprey brain in areas involved with reproduction (9). Furthermore, axons reacting with an antiserum that recognized l-GnRH-I were localized in the median eminence (ME) of humans (10). In the rat, immunoreactive l-GnRH-III neurons and fibers were localized to the dorsomedial preoptic area (POA) with axons projecting to the ME (11, 12). The

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perikarya of these neurons were localized in an area that, upon stimulation, selectively released FSH and, when destroyed, caused a defect in pulsatile FSH, but not LH release (13, 14). This region of the preoptic area contained no m-LHRH neurons. Therefore, considerable evidence indicates that l-GnRH-III is the FSHRH.

In previous studies, we have shown that m-LHRH acts on its receptors to activate a calcium-dependent nitric oxide (NO) pathway that stimulates gonadotropin release from the pituitary gland (15). The adipocyte hormone, leptin, similarly acts on its receptors in the pituitary gland by the NO pathway to produce the release of FSH and LH with equal potency to that of LHRH (15). Therefore, we hypothesized that l-GnRH-III would act on putative FSHRF receptors in the pituitary to activate a calcium-dependent NO pathway to stimulate FSH release. The present experiments were designed to test this hypothesis.

Methods

Chemicals and Animals. Lamprey GnRH-III was synthesized by solid-phase methodology and was purified to more than 99% purity by HPLC in the Protein Facility of Louisiana State University. L-NG-monomethyl-L-arginine (L-NMMA) and ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′,-tetraacetic acid (EGTA) were purchased from Sigma (St. Louis, MO). Adult male rats (200–250 g) of the Sprague-Dawley strain (Holtzman, Madison, WI) were housed under controlled conditions of temperature (23°–25°C) and lighting (lights on from 0500 to 1700 hr). The animals had free access to a pellet diet and tap water.

Mouse GnRH Receptor (GnRHr) Preparation and Inositol Phosphate (IP) Assays. The mouse GnRHr cDNA was shortened by removal of 1.3 kilobases of the 3′-untranslated region by digestion with SspI and was cloned into the EcoRV site of the phagemid pcDNA1/Amp (Invitrogen, San Diego, CA) (16). COS-1 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in a 10% CO_2 atmosphere and were seeded in poly-D-lysine-coated plates at 3 million cells/plate the day before transfection (17) and they were transiently transfected with pcDNA1/Amp-GnRHr constructs (16). Transfected cells were labeled overnight with [H^3]-inositol (2 μCi/ml) in 0.25 ml/well medium 199 containing 5% fetal calf serum, washed twice in HEPES-buffered saline, and then stimulated with synthetic m-GnRH or l-GnRH-III for 60 min in the presence of LiCl (10 m*M*). The reaction was terminated by addition of perchloric acid and phytic acid solution. Total IP were chromatographed on Dowex ion exchange columns and were counted after neutralizing with KOH as described previously (16).

Monolayer Pituitary Cell Cultures. The anterior pituitaries (APs) were obtained from adult male rats after decapitation. Dispersed pituitary cells were cultured as described previously (18). Briefly, the cells were dispersed in Spinner minimum essential medium (MEM) and 100 U/ml penicillin-100 μg/ml streptomycin (PS) with 25 mg of trypsin added. They were centrifuged and resuspended in overnight culture medium (OCM). The cells were counted on a hemocytometer and were diluted in OCM to a final concentration of 2.5×10^5 cells/ml. The cells were cultured with l-GnRH-III or m-GnRH at graded concentrations in medium Medium 199, containing 0.1% bovine serum albumin, 20 m*M* HEPES buffer, and 1 ml of PS (pH 7.4) for 4 days. The cells were then centrifuged, medium pipetted off, and stored frozen for later RIA.

Incubation of Hemipituitaries (HPs) *In Vitro*. Each AP gland of male rats was obtained and bisected longitudinally into two HPs, and each HP was incubated in a vial containing 1 ml of Kreb's-Ringer bicarbonate buffer (KRB) in an atmosphere of 95% O_2-5% CO_2 in a Dubnoff shaker. After 1 hr of preincubation, the medium was replaced with fresh KRB alone or KRB containing graded concentrations of synthetic l-GnRH-III in the presence or absence of NMMA (300 μ*M*) or EGTA (3.0 m*M*) and was incubated for an additional 3 hr. A partially purified FSHRF (fraction 116) from rat hypothalamus was also tested in the 3-hr-incubated HPs. This fraction was obtained by extraction of 1000 rat hypothalami in acetone/0.01N HCl (80:20 vol/vol), purification through Sephadex G-25 column, and bioassays for gonadotropin-releasing activities in rat HPs *in vitro* as previously described (6). The medium was then aspirated and stored frozen until RIA for FSH and LH.

RIAs. The concentrations of FSH and LH in cultured medium were measured by RIAs using kits supplied by the National Institute of Arthritis Digestive Diabetes and Kidney Disease (Dr. A.F. Parlow), and hormone values were expressed as NIH-rFSH-RP-2 and NIH-rLH-RP-3 standards, respectively. The inter- and intraassay coefficients of variation for FSH assays were 6.2% and 5.0%, respectively; and 5.8% and 4.2% for LH assays, respectively.

Statistics. The significance of differences among multiple groups was determined by analysis of variance (ANOVA) with subsequent Newman-Keuls multiple comparisons.

Results

Comparison of Functional Activity of l-GnRH-III and m-LHRH at the m-GnRH mGnRHr in COS-1 Cells. The activity of m-GnRH to its receptor can be determined by the stimulation of labeled IP production. The minimal effective concentration (MEC) for m-LHRH to activate signaling was 10^{-9} *M* and the response reached a maximum at 10^{-7} *M* (Fig. 1). Significant activity of l-GnRH-III was detected only at 10^{-6} *M*, and the maximal response was reached only at 10^{-4} *M*. Thus, the functional activity of l-GnRH-III to activate the m-GnRH receptor was at least 1000 times less than that of m-LHRH.

Monolayer Pituitary Cell Culture. Mammalian LHRH, as reported many times by others (18), was remarkably effective in stimulating both FSH and LH in this assay

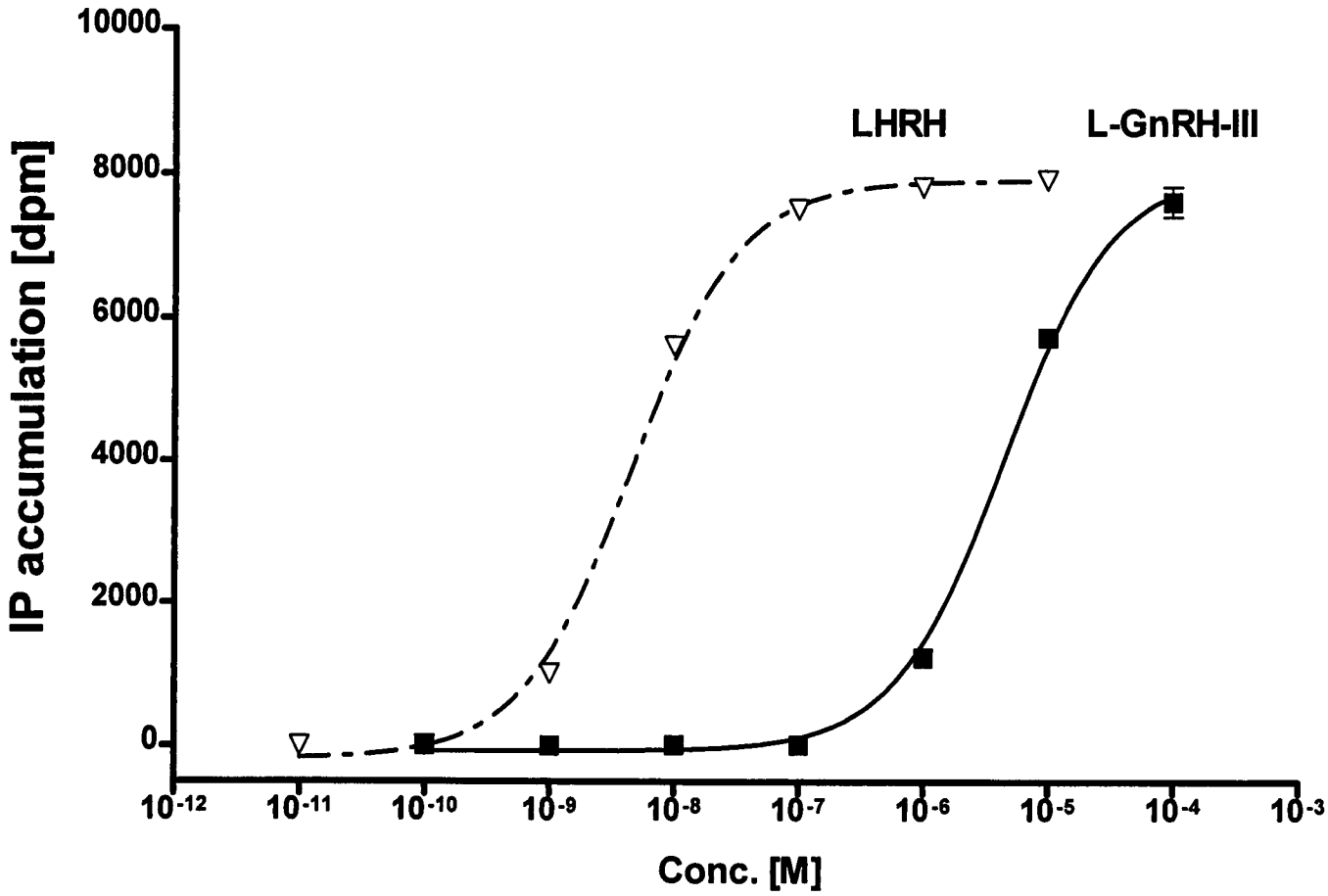

Figure 1. Functional activity of l-GnRH-III at the mouse GnRH receptor. The activity of l-GnRH-III and LHRH at the mouse GnRH receptor was compared in transfected COS-1 cells by assaying the stimulation of IP accumulation. The minimal effective concentration of l-GnRH-III was 10^{-6} M, whereas it was less than 10^{-9} M for LHRH in this assay.

with a highly significant response at the lowest concentration tested (10^{-10} *M*; Figs. 2 and 3). Both responses plateaued at 10^{-8} *M*. The response was much greater for LH than for FSH, namely a 25-fold increase in the release of LH, but only a 4-fold increase in FSH. In contrast, l-GnRH-III was only effective to release LH at a concentration of 10^{-7} *M* and produced a slight increase above the result at the lower concentration when the concentration was increased to 10^{-6} *M*, producing a doubling of the control release. In this assay, l-GnRH-III had little effect on FSH release, the result becoming significant only at 10^{-6} *M* with a small 30% increase, whereas it significantly released LH at 10^{-7} *M*. Therefore, both m-LHRH and l-GnRH-III in this assay were more effective in releasing LH than FSH. As in the case of the m-LHRH receptor assay, l-GnRH-III was at least 1000 times less potent than m-LHRH.

Hemianterior Pituitaries Incubated *In Vitro*. In striking contrast to the results shown with the monolayer cultured cells, l-GnRH-III exerted preferential FSH-releasing activity in the hemianterior pituitary system (Fig. 4), but the minimal effective dose was not determined; however, the activity was highly significant at 10^{-8} and 10^{-7} *M*, whereas neither of these concentrations significantly stimulated LH release (Fig. 5), confirming the specificity of l-GnRH-III in releasing FSH selectively in the HP assay.

Effects of Calcium on Gonadotropin-Releasing Activity of l-GnRH-III. The FSH releasing activity of l-GnRH-III (10^{-8} and 10^{-7} *M*) was completely blocked by removal of calcium from KRB buffer and adding EGTA (3 m*M*), a calcium chelating agent (Fig. 4). In this experiment, l-GnRH-III only stimulated LH release at the higher dose of 10^{-7} *M*, and this activity was also blocked by removal of calcium (Fig. 5). In the two KRB control groups, removal of calcium and addition of EGTA led to a slight but not significant decrease ($P = 0.06$) of LH, but not FSH release from the pituitaries.

Effects of NMMA on l-GnRH-III and FSHRF Activity. The FSH-releasing activity of l-GnRH-III (at doses of 10^{-8} and 10^{-7} *M*) was blocked by NMMA (300 μ*M*; Fig. 6), whereas l-GnRH-III alone (10^{-8} and 10^{-7} *M*) or the peptide together with NMMA (300 μ*M*) had no effect on LH release *in vitro* (Fig. 7). L-NMMA alone did not alter basal FSH or LH release (Figs. 6 and 7). The highly significant release of FSH ($P < 0.01$) induced by partially purified FSHRF was also completely blocked by NMMA ($P < 0.001$; Fig. 8).

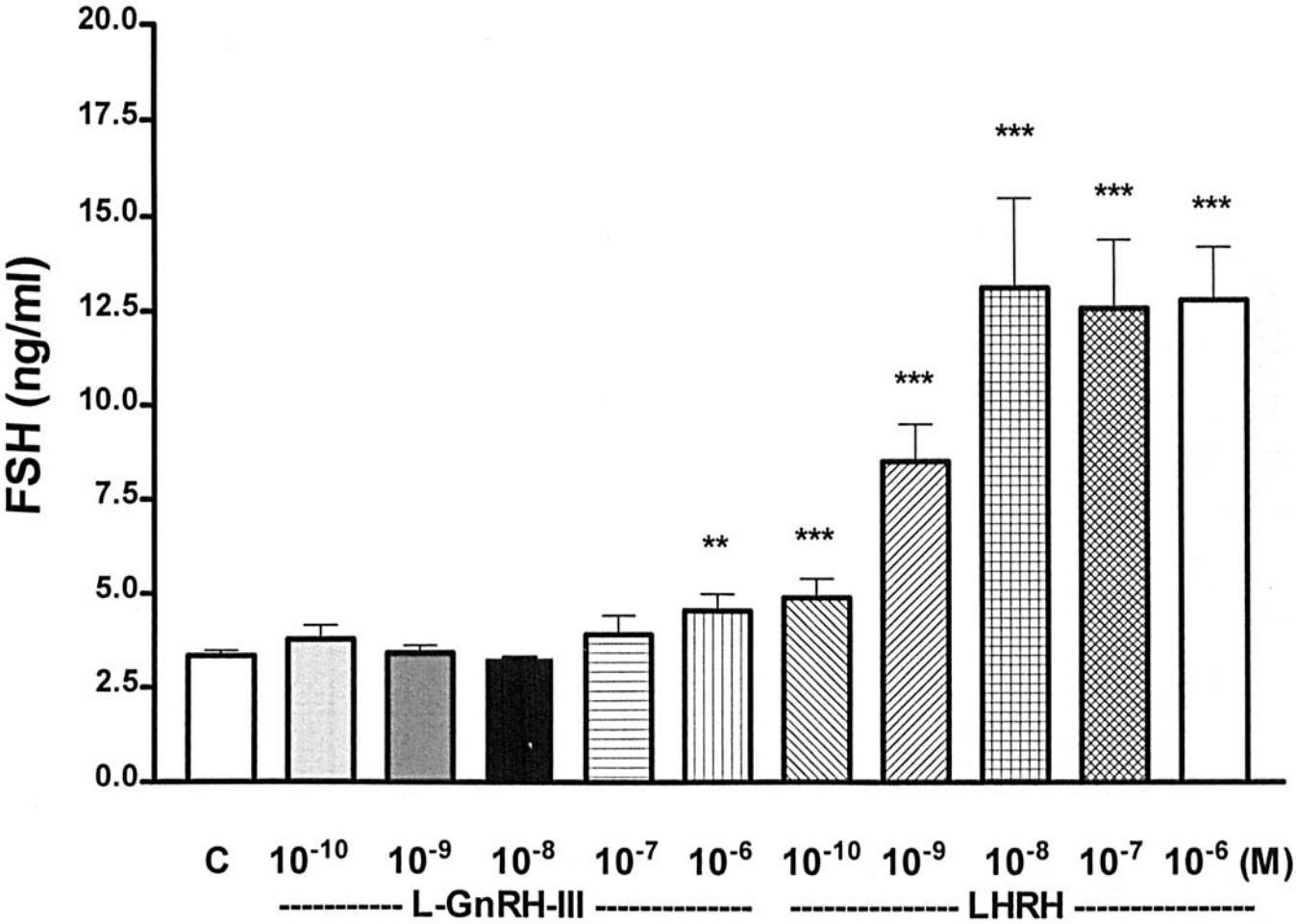

Figure 2. Effects of l-GnRH-III and LHRH on the release of FSH from 4-day monolayered cultured anterior pituitary cells of male rats. l-GnRH-III stimulated FSH release only at the high dose of 10^{-6} M, whereas LHRH increased FSH release at doses of 10^{-10} to 10^{-6} M. Values represent means ± SEM. C, control group. $n = 6$ in each group. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs controls.

Discussion

These results clearly show that l-GnRH-III releases FSH selectively when incubated with hemianterior pituitaries, confirming our previous results. It also has a preferential FSH-releasing activity in the ovariectomized estrogen progesterone-blocked rat (7) and in the normal male rat (6, 7). Our recent studies have shown that it similarly has a selective FSH-releasing activity in cows at the appropriate stage of the cow estrous cycle (19).

The results under these conditions differed dramatically from those obtained in 4-day monolayer cultured cells. Here, we show that l-GnRH-III has essentially no selective FSH-releasing activity in this assay, and furthermore, even with increasing doses, there was only a small stimulation of FSH release. Similarly, l-GnRH-III had a very high MEC to activate IP formation in COS cells transfected with m-LHRH receptors. Thus, the responsiveness of the monolayer cultured cells to l-GnRH-III can be accounted for solely by its binding to m-LHRH receptors on these cells. This also is consistent with the behavior of l-GnRH-III on monolayer cultured cells, which was similar to that of m-LHRH with a greater release of LH than FSH. Thus, the potency of l-GnRH-III on m-LHRH receptors and monolayer cultured cells was 1000 times less than that of m-LHRH.

These results are opposite to those in the HP situation in which sensitivity for FSH release was 10^{-9} *M*, and a much higher dose was required for LH release *in vitro* in the hemianterior pituitaries (7) and in the present results. l-GnRH-III also selectively released FSH *in vivo* in ovariectomized, estrogen progesterone-blocked rats (7) and in normal male rats (Yu *et al.*, unpublished data) as well as in the cow in the progestational phase of its cycle (19). Therefore, we suggest that the failure of l-GnRH-III to release FSH selectively in the 4-day monolayer cultured cells is related to the absence of gonadal steroids from the medium, resulting in downregulation of FSHRF receptors to the vanishing point. On the other hand, the LHRH receptors are maintained or possibly enhanced. We hypothesize that in the normal animal, FSHRF receptors are present, and that they are augmented by estrogen and possibly also by progesterone in the ovariectomized females to account for the much greater sensitivity of these animals to FSHRF and l-GnRH-III

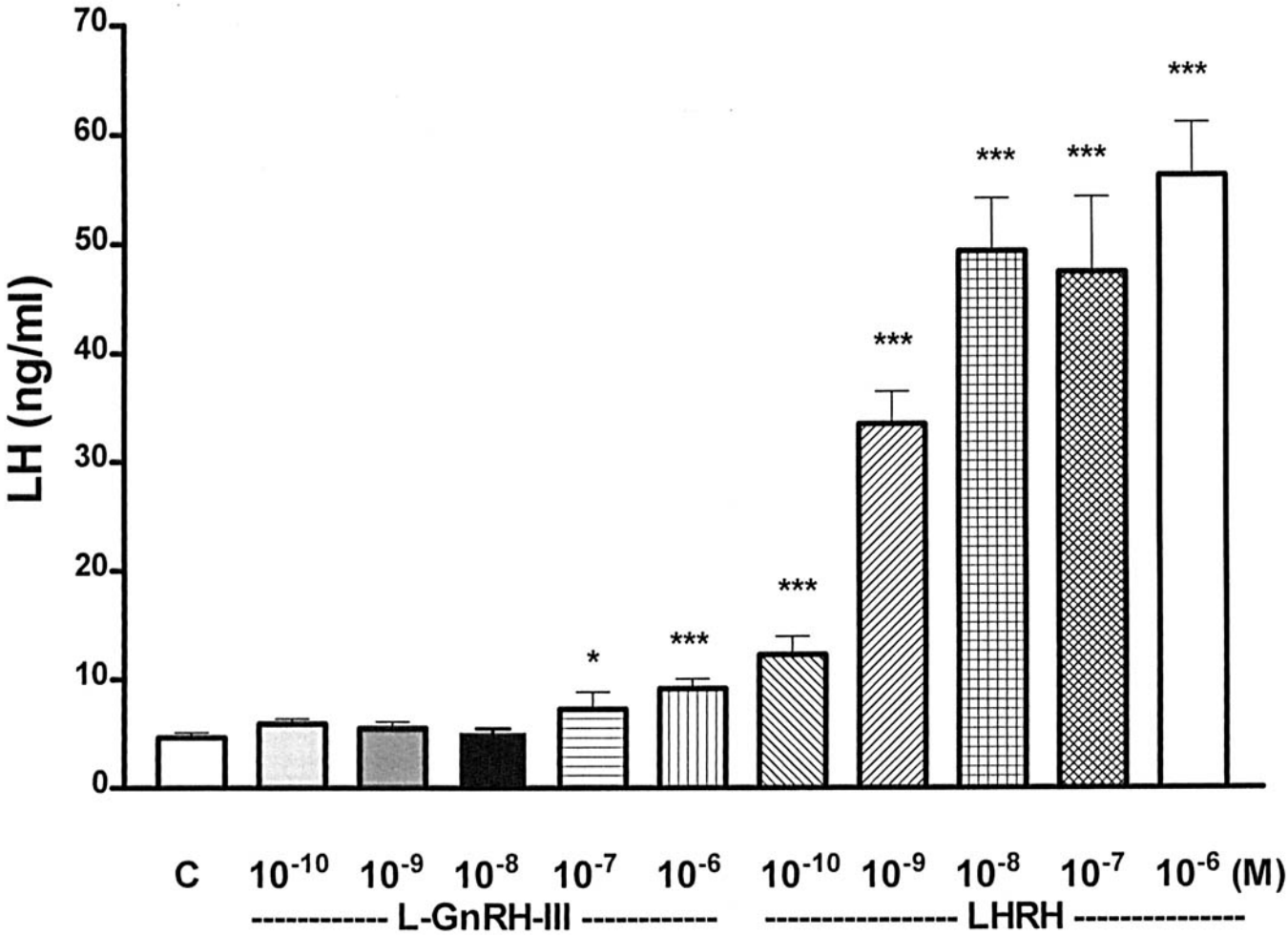

Figure 3. Effects of l-GnRH-III and LHRH on the release of LH from 4-day monolayered cultured pituitary cells. l-GnRH-III stimulated LH release at doses of 10^{-7} and 10^{-6} M, whereas LHRH stimulated LH release at all doses tested (10^{-10} to 10^{-6} M). $n = 6$ in each group. C, control group. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs controls.

than that of normal animals (7). The LHRH receptors are also upregulated by estrogen and progesterone, accounting for the super-sensitivity not only to FSHRF, but also LHRH of ovariectomized estrogen, progesterone-treated rats (7).

Supporting the concept of a separate l-GnRH-III receptor on the gonadotropes, a biotinylated l-GnRH-III, which has selective FSH-releasing activity when tested in the HP assay, was used to identify l-GnRH-binding sites on gonadotropes acutely dispersed from male rat pituitaries. Indeed, biotinylated l-GnRH-III bound preferentially to FSH gonadotropes at a concentration of 10^{-9} *M*. The binding could not be displaced by m-LHRH. The concentration to demonstrate binding was the same as that active to release FSH from HP. If the dispersed cells were used 24 hr after dispersion, the binding was greatly reduced, results that suggest that even within 24 hr, the FSHRF receptors are being downregulated in the absence of steroids, fitting with the lack of selective FSHRF activity of l-GnRH-III in the 4-day monolayered cultured cells alluded to above (20). Therefore, the accumulated evidence from these various approaches suggests that an FSHRF receptor will ultimately be identified on the FSH gonadotropes.

Recently, a GnRH-II receptor has been characterized by two groups from primate pituitaries (21, 22) and its ligand, chicken (c) GnRH-II, has been identified in brains of mammals (23) including primates (24, 25). It has been suggested that c-GnRH-II may be FSHRF because, under certain conditions, it provokes greater FSH than LH release (22). Indeed, we discovered this effect testing c-GnRH-II in ovariectomized, estrogen progesterone-blocked rats earlier (5); however, the pattern of FSH release differs from that of l-GnRH-III in that the minimal effective dose (MED) for FSH and LH release with c-GnRH-II is almost the same, whereas with l-GnRH-III, as indicated, there is a much lower MED for FSH release than for LH release. Also, c-GnRH-II had only weak activity in the HP assay and no selectivity to release FSH (7). Lastly, we have not been able to detect appreciable c-GnRH-II in the rat hypothalamus using an antiserum that readily demonstrates it in the chicken brain (11, 12). c-GnRH-II may not reach the pitu-

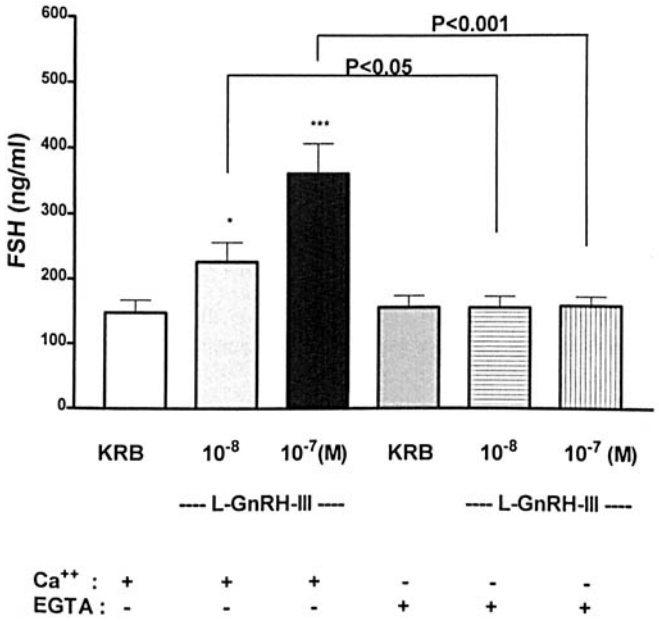

Figure 4. The FSH-releasing activity of I-GnRH-III in anterior HPs *in vitro* was blocked by removing calcium from KRB buffer and adding EGTA (3.0 m*M*), a chelating agent. Values represent means ± SEM. $n = 8$ in each group. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs KRB controls.

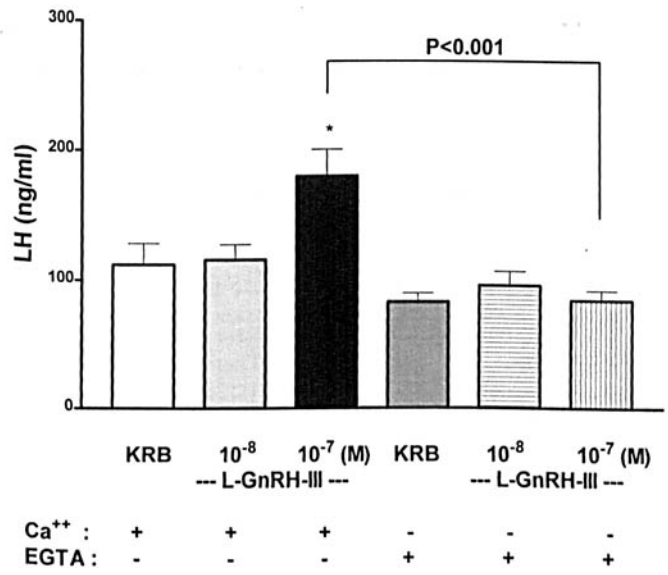

Figure 5. L-GnRH-III only stimulated LH release from anterior HPs *in vitro* at the dose of 10^{-7} M ($P < 0.05$), and this activity was blocked by removing calcium from the buffer. $n = 8$ in each group. $^{*}P < 0.05$ vs KRB (with the presence of calcium) controls.

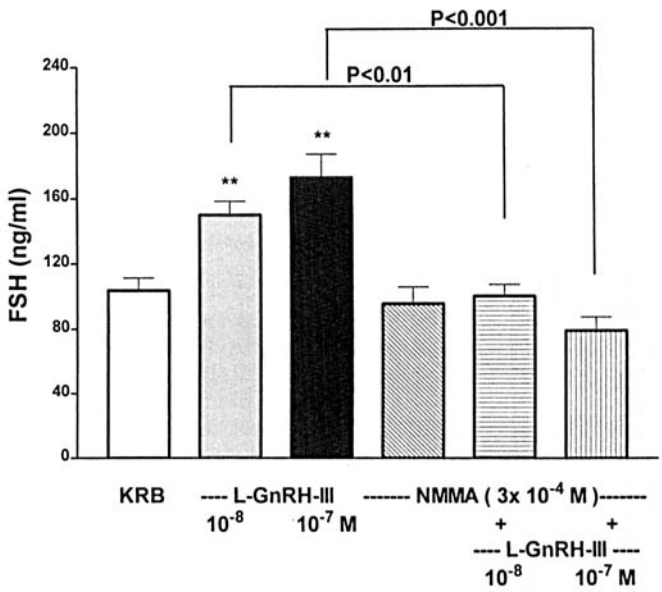

Figure 6. The FSH-releasing activity of I-GnRH-III from incubated anterior HPs was blocked by N^{G}-monomethyl-L-arginine (NMMA, an inhibitor of NO synthase [NOS]). NMMA itself had no effect on FSH release at the dose of 3×10^{-4} M. Values represent means ± SEM. $n = 7$ in each group. $^{**}P < 0.01$ vs KRB controls.

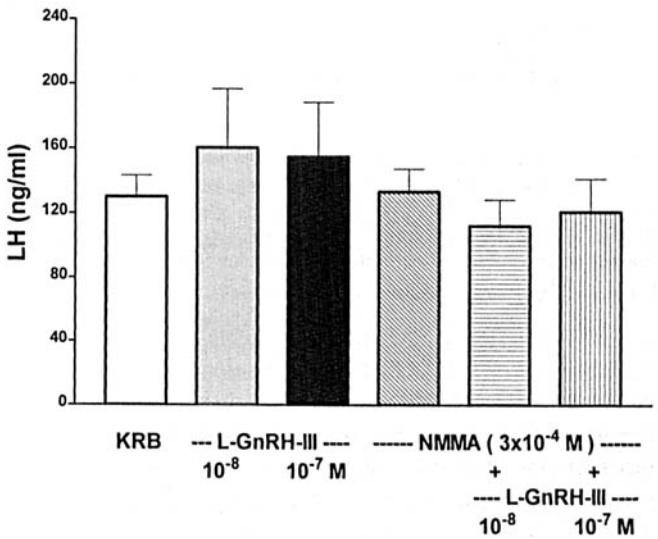

Figure 7. I-GnRH-III alone (10^{-8} and 10^{-7} M) or this peptide with NMMA together had no effect on LH release from anterior HPs incubated *in vitro*. $n = 7$ in each group.

itary because it has not been shown to be present in significant amounts of the ME of rats (12). I-GnRH-III activates GnRH-II receptors, but only at a MEC of 10^{-6} *M* (J.D. Neill, personal communication), results that support the probability that I-GnRH-III must activate a putative FSHRF receptor rather than either of the previously described GnRH-I or II receptors.

It is well known that FSH and LH release is controlled by calcium ions (Ca^{++}) (26, 27) and that interaction of LHRH with its receptor causes an increase in intracellular free calcium and also activates the phosphatidyl inositol cycle that mobilizes internal calcium. The resulting increase in intracellular free calcium mediates the releasing action of LHRH (27); however, we earlier showed a role for cGMP and not cAMP in controlling the release of LH and FSH mediated by LHRH (28–30). This was before it was accepted that NO is a physiologically significant, gaseous transmitter that acts by activation of guanylyl cyclase that converts GTP to cGMP. cGMP activates protein kinase G that causes exocytosis of gonadotropin secretory granules.

To test the hypothesis that the FSH-releasing activity of I-GnRH-III (or FSHRF) is regulated by calcium and NO, calcium was removed from the medium and a chelating agent EGTA that would remove any residual Ca^{++} was

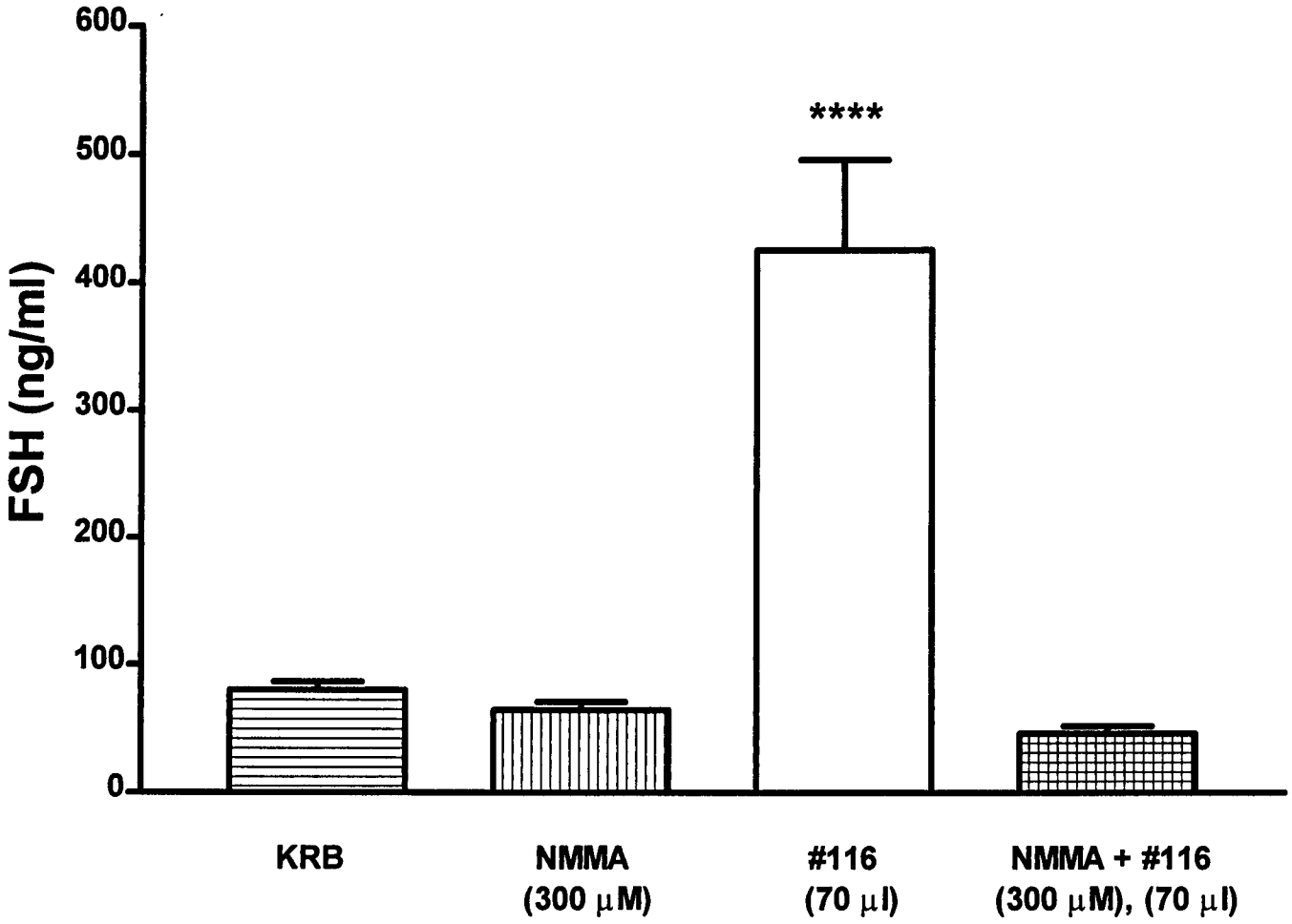

Figure 8. NMMA completely blocked ($P < 0.001$) the FSH-releasing activity of purified rat FSHRF (tube 116 of Sephadex-purified rat stalk-median eminence extract) from anterior HPs incubated *in vitro*. $n = 7$ in each group.

added. The action of FSHRF and l-GnRH-III was blocked in the absence of Ca^{++}. NMMA, a competitive inhibitor of NOS, was added to the medium in other experiments. We found that the inhibitor of NOS, NMMA, completely blocked the FSH-releasing activity of not only purified FSHRF (Fig. 8, fraction 116) but also of l-GnRH-III. Furthermore, sodium nitroprusside (NP), a releaser of NO, stimulated both LH and FSH release, and the activity of LHRH to release both LH and FSH was also blocked by NMMA. These data indicate that FSHRF (or l-GnRH-III) acts on its putative receptor via a calcium-dependent, NO pathway to release FSH specifically, whereas LHRH acts on its receptor similarly to increase intracellular Ca^{++} that activates NOS in the gonadotrophs to cause release of LH and to a lesser extent FSH (15).

Thus, our results provide further support for the concept that l-GnRH-III is FSHRF that is responsible for selective release of FSH at the time of initiation of pubertal development and that it accounts for the differential pulsing of FSH and LH in the rat. It, like m-LHRH, acts on its receptors by increasing intracellular Ca^{++}, which activates nNOS in the gonadotropes. The NO released activates guanylyl cyclase, causing production of cGMP from GTP that, by protein kinase G, causes exocytosis of FSH secretory granules from FSH gonadotropes. The results have been reported in part in abstract form (31).

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