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Potent Action of RFamide-Related Peptide-3 on Pituitary Gonadotropes Indicative of a Hypophysiotropic Role in the Negative Regulation of Gonadotropin Secretion

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We identified a gene in the ovine hypothalamus encoding for RFamide-related peptide-3 (RFRP-3), and tested the hypothesis that this system produces a hypophysiotropic hormone that inhibits the function of pituitary gonadotropes. The RFRP-3 gene encodes for a peptide that appears identical to human RFRP-3 homolog. Using an antiserum raised against RFRP-3, cells were localized to the dorsomedial hypothalamic nucleus/paraventricular nucleus of the ovine brain and shown to project to the neurosecretory zone of the ovine median eminence, predicating a role for this peptide in the regulation of anterior pituitary gland function. Ovine RFRP-3 peptide was tested for biological activity *in vitro* and *in vivo*, and was shown to reduce LH and FSH secretion in a specific manner. RFRP-3 potently inhibited GnRH-stimulated mobilization of intracellular calcium in gonadotropes. These data indicate that RFRP-3 is a specific and potent mammalian gonadotropin-inhibiting hormone, and that it acts upon pituitary gonadotropes to reduce GnRH-stimulated gonadotropin secretion. (*Endocrinology* 149: 5811–5821, 2008)

GnRH IS THE PRIMARY stimulator of gonadotropin secretion (1), but there is mounting evidence of a functional inhibitory hormone in the hypothalamus (2, 3). Such an entity has been sought for many years (4), with definitive evidence of the existence of gonadotropin inhibitory hormone (GnIH) first obtained in the avian brain (5). Data have since accumulated to indicate that GnIH negatively regulates reproductive function in hamsters and rats (6, 7). Most of the focus in mammalian studies has been on central actions of GnIH to inhibit GnRH function, but there is also an indication that GnIH acts on pituitary gonadotropes in birds (5, 8–11). The GnIH receptor is also expressed in the avian pituitary and brain (11, 12), predicating a role for the hormone as a hypophysiotropic factor.

There are various homologs of GnIH (for review, see Ref. 13) that belong to a large family of peptides with a common Arg-Phe-NH_2 C terminus (RF-amide) (for review, see Ref. 14). We now report the existence of an ovine gene encoding for RFamide-related peptide-3 (RFRP-3) (GnIH). We localized RFRP-3 cells in the ovine hypothalamus and determined that the cells project to the neurosecretory zone of the median eminence. Physiological studies on pituitary gonadotropes then revealed specific and potent effects to reduce gonadotropin secretion, by counteracting the effect of GnRH to increase intracellular free calcium.

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Abbreviations: AUC, Area under the curve; AVP, vasopressin; BDA, biotinylated dextran amine; DMN, dorsomedial hypothalamic nucleus; GnIH, gonadotropin inhibitory hormone; ORX, orexin; OT, oxytocin; PSS, physiological saline solution; PVN, paraventricular nucleus; RFRP-3, RFamide-related peptide-3; SSC, standard saline solution.

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Materials and Methods

All animal procedures were conducted with prior institutional ethical approval under the requirements of the Australian Prevention of Cruelty to Animals Act 1986, and the National Health and Medical Research Council/Commonwealth Scientific and Industrial Research Organization/Australian Animal Commission *Code of Practice for the Care and Use of Animals for Scientific Purposes*.

Animals

The animals used in this study were female Corriedale ewes of 3–5 yr, that were ovariectomized to remove the influence of gonadal steroids. When required, the animals were euthanized with an iv injection of 20 ml Lethobarb (pentobarbitone sodium 325 mg/ml; Virbac, Peakhurst, New South Wales, Australia). For the purpose of blood sampling, animals were contained in single pens, within an animal facility subject to natural light and temperature. When confined, the animals received chaffed lucerne hay and water *ad libitum*. Otherwise, the animals were maintained on pasture with supplemental feeding of meadow hay.

Gene cloning

A partial sequence of the ovine GnIH precursor gene was generated by RT-PCR cloning using ovine hypothalamic cDNA extract and primers based on the known bovine RFRP sequence (GenBank accession no. NM_174168) (15), as shown in Fig. 1.

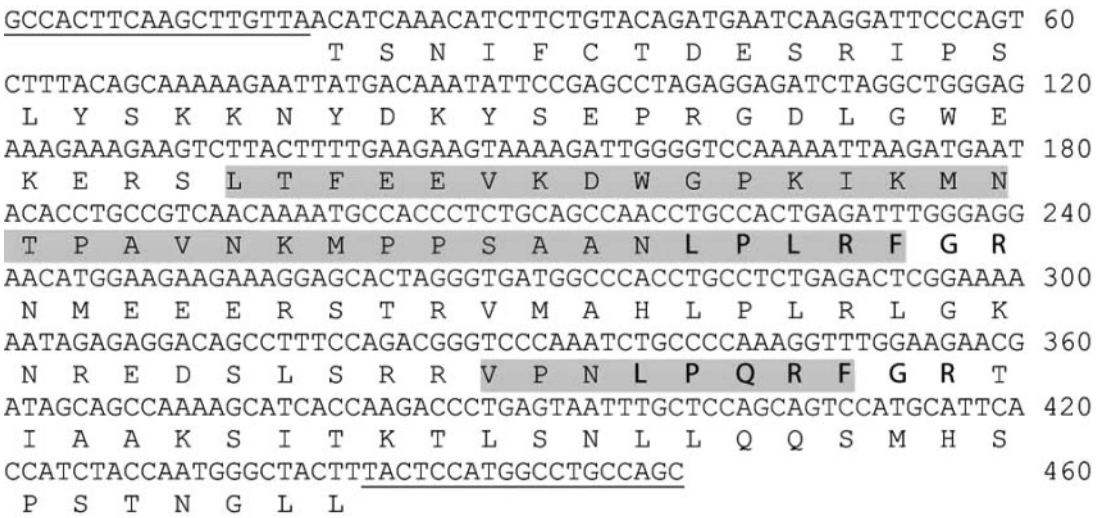

FIG. 1. Ovine GnIH precursor cDNA sequence and its deduced amino acid sequence. Ovine RFRP-1 could be 34 or 12 amino acids, depending on where it is cleaved (*first highlighted sequence*). Sheep RFRP-3 is also *highlighted* (VPNLPQRF), and is identical to human and bovine RFRP-3 (15, 29). This ovine cDNA has 93% common identity with bovine RFamide-related precursor polypeptide (accession no. AB040291) The C-terminal amino acid sequences for ovine GnIH peptides [-LPXRF-amide (X = L or Q)], including glycine as an amidation signal and arginine as an endoproteolytic basic amino acid, are shown in *bold*. *The underlined sequences* are the forward and reverse primers used for PCR.

In situ hybridization and immunohistochemistry

The regional localization of GnIH cells in the ovine hypothalamus was mapped by *in situ* hybridization, and further definition of localization was obtained by labeling the same sections with antisera that define locality to the paraventricular nucleus (PVN)/dorsomedial hypothalamic nucleus (DMN). For the former we used a monoclonal neurophysin antiserum that identifies oxytocin (OT) and vasopressin (AVP) cells (PS45) (gift from Dr. H Gainer, Molecular Neuroscience Section, Lab of Neurochemistry, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD) (16). For the latter we used a rabbit polyclonal antiserum against orexin (ORX) (17) that we have used previously for ovine brain (18). This neuropeptide is produced in the dorsomedial nucleus (18).

This combined *in situ* hybridization and immunohistochemistry procedure was conducted on free-floating paraformaldehyde-fixed sections (40 μm) of ovine hypothalamus, prehybridized for 2 h at 58 C in a hybridization buffer [50% formamide, 5× standard saline citrate (SSC) (pH 7.0), 250 μg/ml herring sperm DNA, 100 μg/ml yeast tRNA, 100 μg/ml heparin, 5% dextran sulfate, 1× Denhardt's solution, and 0.1% Tween 20]. The probe was then added to a final concentration of 100–250 ng/ml, and sections were incubated at 58 C overnight. After hybridization the sections were washed 2 × 30 min in 2× SSC, 0.1% Tween 20 at 58 C, followed by 2 × 30 min in 0.1× SSC, 0.1% Tween 20 at 58 C. The sections were rinsed in maleic acid buffer 2 × 15 min (0.1 M maleic acid, 0.15 M NaCl, and 0.1% Tween 20) at room temperature, and incubated for 2 h in maleic acid buffer containing 2% Boehringer Blocking Reagent (Hoffmann-La Roche Inc., Nutley, NJ), 10% goat serum, and 2 mM levamisole. A mixture of three primary antibodies, including alkaline phosphatase conjugated sheep anti-digoxigenin antibody (1:1000; Hoffmann-La Roche), PS45 (neurophysin) (1:5000), and rabbit anti-ORX (dilution 1:2000) was then applied for 48–72 h at 4 C. The sections were washed in 10 mM Tris-HCl buffer with 0.9% NaCl (pH 7.4), and the PS45 and ORX labeling was visualized with goat antimouse Alexa 488 and goat antirabbit Alexa 546, respectively (1:500; Molecular Probes, Inc., Eugene, OR). To visualize the digoxigenin-labeling, the sections were washed 3 × 1 h in maleic acid buffer with 2 mM levamisole. This was followed by 2 × 15 min equilibration steps in alkaline buffer [0.1 M NaCl, 0.1 M Tris-HCl (pH 9.5), 0.1 M $MgCl_2$, 0.1% Tween 20, and 2 mM levamisole] with nitroblue tetrazolium. Then 5-bromo-4-chloro-3-indolyl phosphate salts (Hoffmann-La Roche) were applied to reveal the digoxigenin-labeled neurons. The sections were then mounted on gelatin-chrome coated slides and coverslipped with antifade fluorescent medium (Dako Corp., Botany, New South Wales, Australia). A negative control (exclusion of primary antibody) was included for each immunohistochemical run.

For *in situ* hybridization with ^{35}S label, the 460-base cDNA sequence shown in Fig. 1 was inserted into a pGemT-easy plasmid. Under a standard transcription protocol, antisense and sense ovine GnIH riboprobes were transcribed with T7 and SP6 polymerase, respectively (Promega Corp., Madison, WI), with ^{35}S-uridine 5′-triphosphate (GE Healthcare Life Sciences, Boston, MA). The riboprobe was separated from unincorporated nucleotides on a Sephadex G-25 column (Sigma-Aldrich Corp., St. Louis, MO). No signal was observed after the application of sense probe (data not shown). This was applied to sections using a protocol previously described (19).

Anterograde tracing from the PVN to the median eminence

A polyclonal antiserum was raised in guinea pig using RFRP-3 conjugated to keyhole limpet hemocyanin as an immunogen (no. 1354; Antibodies Australia, Melbourne, Australia). The titer (1:4000) and specificity of this antiserum were tested by immunohistochemistry on sections of formalin-perfused tissues prepared as described previously (20). Coronal sections (40 μm) of the hypothalamus were cut on a cryostat and prepared for immunohistochemistry as described. Sections that included the region of the PVN and DMN were incubated with the antiserum at a dilution of 1:4000, with or without 0.1 mg/ml RFRP-3, quail GnIH, hamster GnIH, kisspeptin, nuclear form factor, prolactin-releasing peptide, chemerin, GnRH, and QRFP43. RFRP-3, and quail and hamster GnIH abolished staining, whereas the other peptides did not reduce the intensity of immunostaining (data not shown).

To determine whether GnIH cells of the PVN/DMN region project to the external zone of the median eminence, we injected 50–70 μl biotinylated dextran amine (BDA) into three sheep, using methods previously described (20). The animals were euthanized after 3 wk, and hypothalamic blocks were processed for immunohistochemistry. One animal with an injection that was clearly localized to the central region of the PVN was selected for detailed study. BDA was detected by avidin-fluorescein isothiocyanate (1:500), and GnIH immunostaining was visualized with Alexa 546 (1:500). Images of the median eminence were merged to determine whether anterograde tracer and GnIH were present in neuronal terminals in the external neurosecretory zone.

In vitro analysis of the effect of RFRP-3

Pituitaries were obtained from sheep immediately after euthanasia and placed in sterile DMEM solution. The tissues were minced and the cells dissociated by incubation (25 min at 37 C) in 10 ml DMEM plus 0.5% BSA/PBS. This solution contained (per 20 ml) collagenase (0.03 g), deoxyribonuclease (200 μl), hyaluronidase (200 μl), trypsin inhibitor

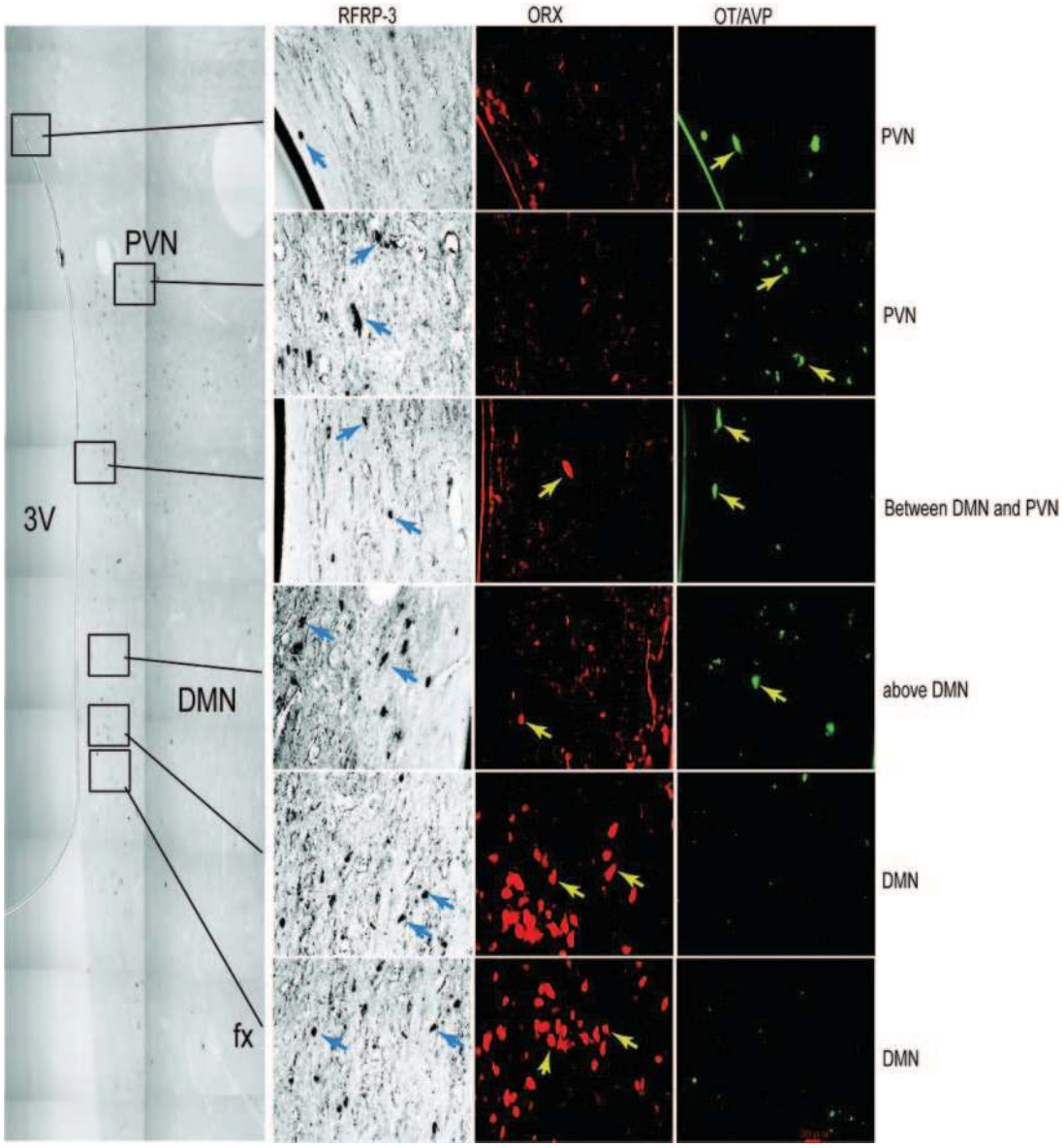

FIG. 2. Combined *in situ* hybridization localization of RFRP-3 cells and immunohistochemical localization of ORX and OT and AVP cells (OT plus AVP) cells in the ovine brain. A series of sections in the region of the PVN and DMN were used to localize RFRP-3 by *in situ* hybridization with digoxigenin label (*left column*). The same sections were then immunostained for ORX (*middle column*, red Alexa 546 visualization) and neurophysin to localize OT and AVP cells (*right column*, green Alexa 488 visualization). ORX defines the region of the DMN, and OT/AVP defines the region of the PVN. *Arrows* indicate examples of labeled cells. 3V, Third ventricle. *The boxed areas* in the left hand image represent various regions of the PVN and DMN and are enlarged in the three right hand columns. Fx, fornix.

(200 μl), and pancreatin (500 μl). The cells were triturated, and the process was repeated twice at 5-min intervals. After passing through mesh and centrifugation, the cells were resuspended in 50 ml 0.1% BSA/PBS. The percentages of live cells (>95%) were estimated by Trypan blue exclusion. Finally, the cells were pelleted and resuspended in DMEM containing fetal calf serum (10%), including antibiotic (1% antimycotic-penicillin), and plated out at a density of 0.5×10^6 per well in 1 ml DMEM containing 10% fetal calf serum and antibiotic for 72 h before experimentation. On the day of experimentation, the medium was removed and replaced with serum free DMEM containing 0.1% BSA and allowed to equilibrate for 2 h. This medium was then replaced with medium containing either GnRH (10^{-9} M) or GnRH plus RFRP-3 (Val-Pro-Asn-Leu-Pro-Gln-Arg-Phe-NH_2) that was synthesized by conventional solid-phase methodology and purified on preparative HPLC. The peptide was 95% pure on analytical HPLC. The molecular mass was confirmed by mass spectrometry. RFRP-3 was used at doses of 10^{-8} to 10^{-16} M with six wells per treatment. The supernatant was collected 2 h later, and assayed for LH and FSH by RIA. These experiments were replicated three times with similar results, although data are presented for only one of the replicates. To determine the specificity of the effect on LH and FSH secretion, we treated cells with a non-amidated form of GnIH, using the same protocol as described previously. In this case a non-amidated hamster GnIH was available so the experiments were performed with amidated and non-amidated hamster peptide (21).

Effect of RFRP-3 on GnRH-stimulated generation of intracellular free calcium

Cells obtained from ovariectomized ewes as described previously were allowed to settle on glass coverslips (9 mm diameter) at 35 C, in an atmosphere containing 5% CO_2 and at 85% humidity. The cells were used 12–48 h after plating. The cells were washed with physiological saline solution

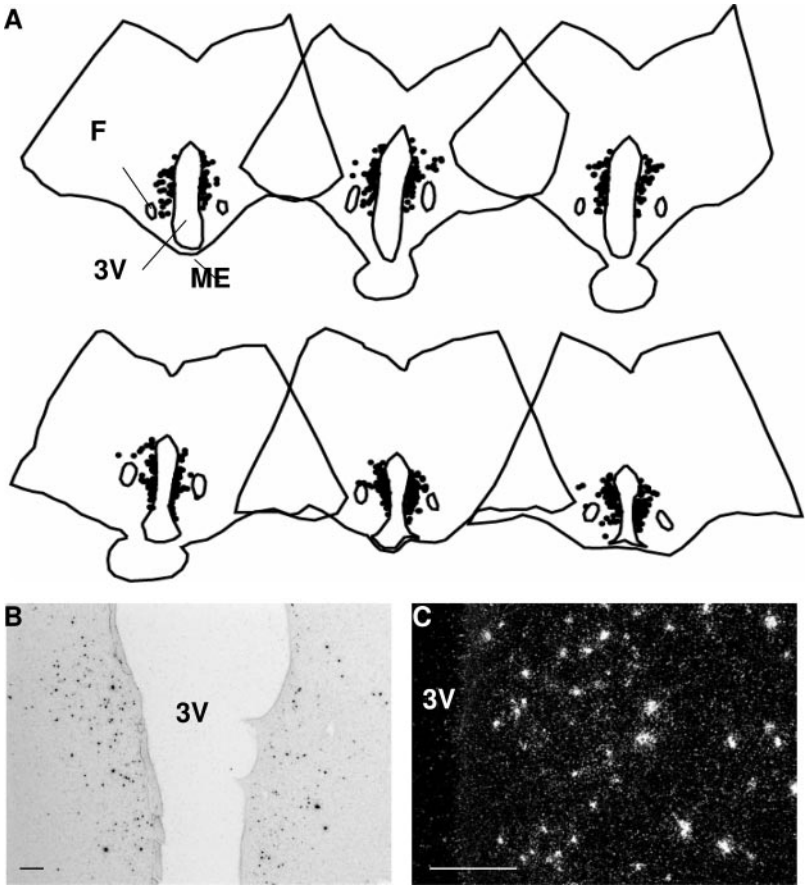

FIG. 3. *In situ* hybridization with ^{35}S-labeled riboprobe, indicating the location of RFRP-3 expressing cells in the PVN and DMN. A, Cells mapped to this region. B, Low-power expression (*scale bar*, 200 μm) of cells visualized by *in situ* hybridization. C, A higher power (*scale bar*, 200 μm) view of cellular labeling. F, Fornix; ME, median eminence; 3V, third ventricle.

(PSS) containing (mM): NaCl 117, KCl 5, $MgCl_2$ 2, KH_2PO_4 0.5, $NaHCO_3$ 5, HEPES 10, glucose 10, and BSA 0.1%, at pH 7.35, and were then loaded with Fluo-4-AM (Sigma-Aldrich) (5×10^{-6} M) in PSS containing 0.01% pluronic for 30 min at room temperature (23 C) in the dark. Coverslips with loaded cells attached were transferred to a Warner tissue bath (Warner Instruments, LLC, Hamden, CT) mounted on an Olympus IX71 inverted microscope (Olympus, Hamburg, Germany) and continuously superfused with PSS at 33 C for 20 min to allow cleavage of Fluo-4-AM by cytoplasmic esterases. The cells were viewed under confocal conditions during which they were illuminated by a krypton/argon laser at 488 nm, and the light passed through a Yokogawa CSU22 Nipkow spinning disc (Yokogawa Australia Pty. Ltd., Macquarie Park New South Wales, Australia) to a high-sensitivity electron-multiplying Andor iXon CCD camera (Andor Technology PLC, Belfast, Northern Ireland). Full frames were collected at 2-sec intervals. Cells that responded to GnRH with an increase in cytoplasmic calcium were accepted as gonadotropes. These cells had low, stable cytoplasmic levels in the absence of stimulation and responded to GnRH with a large spike in cytoplasmic calcium that was sustained for approximately 30 sec. Gonadotropes thus defined constituted approximately 10% of pituitary cells. Cytoplasmic calcium fluctuated spontaneously in an additional 10% of cells, but neither the amplitude nor the frequency of these oscillations was changed by GnRH or RFRP-3. RFRP-3 did not alter cytoplasmic calcium in cells that were unresponsive to GnRH. After 5 min control recording, the cells were exposed to 2×10^{-9} M GnRH for 1 min (exposure 1, to identify gonadotropes), followed by a 30-min period of superfusion without GnRH. The cells were then challenged with GnRH for a second time at 35 min (exposure 2). Either vehicle or RFRP-3 (10^{-8} to 10^{-14} M) was applied for 20 min before and during exposure 2, and the laser was turned off in this period to prevent photobleaching of the cells. This was followed by a 1-min application of PSS containing 30 mM KCl (isosmotic substitution of NaCl) at 40 min to confirm integrity of the cells. The experiment included replicates from eight sheep for vehicle treatment and three to four sheep for RFRP-3 treatment.

Effect of RFRP-3 on pituitary hormone release in vivo

Adult ewes were used at least 1 month after ovariectomy. The animals received bilateral jugular venous cannulae for sampling and infusion of human RFRP-3 (n = 3) or saline vehicle (n = 3). Blood samples were collected at 10-min intervals over 5 h. At 120 min the ewes received an initial priming dose of 50 μg human RFRP-3, followed by a continuous infusion of 200 μg over 1 h. Blood samples were rapidly transferred to heparinized tubes on ice and then centrifuged at 4 C. Plasma was stored at −20 C until assayed by RIA to measure the levels of LH and FSH. To determine the specificity of the response, plasma levels of GH, prolactin, and cortisol were also measured.

RIAs

Established methods were used for the measurement of LH (22), FSH (23), GH (24), prolactin (25), and cortisol (26).

Data Analysis

Treatment means for *in vitro* data were analyzed by ANOVA. For analysis of *in vivo* hormone data, the mean area under the curve (AUC) was calculated before and during infusion of RFRP-3, and LH pulse amplitude was also calculated (27). These data were then analyzed by ANOVA. We used Scheffé's test for *post hoc* comparisons.

For the measurement of cytoplasmic free calcium, responses for single cells to GnRH were expressed as: (change in fluorescence exposure 2) × 100/(change in fluorescence exposure 1).

The mean values from 10–20 cells for each sheep were used for statistical analysis. The least squares method was used to construct a sigmoid concentration response curve, and to determine the maximum amplitude of exposure 2 (E_{max}) in response to GnRH and the

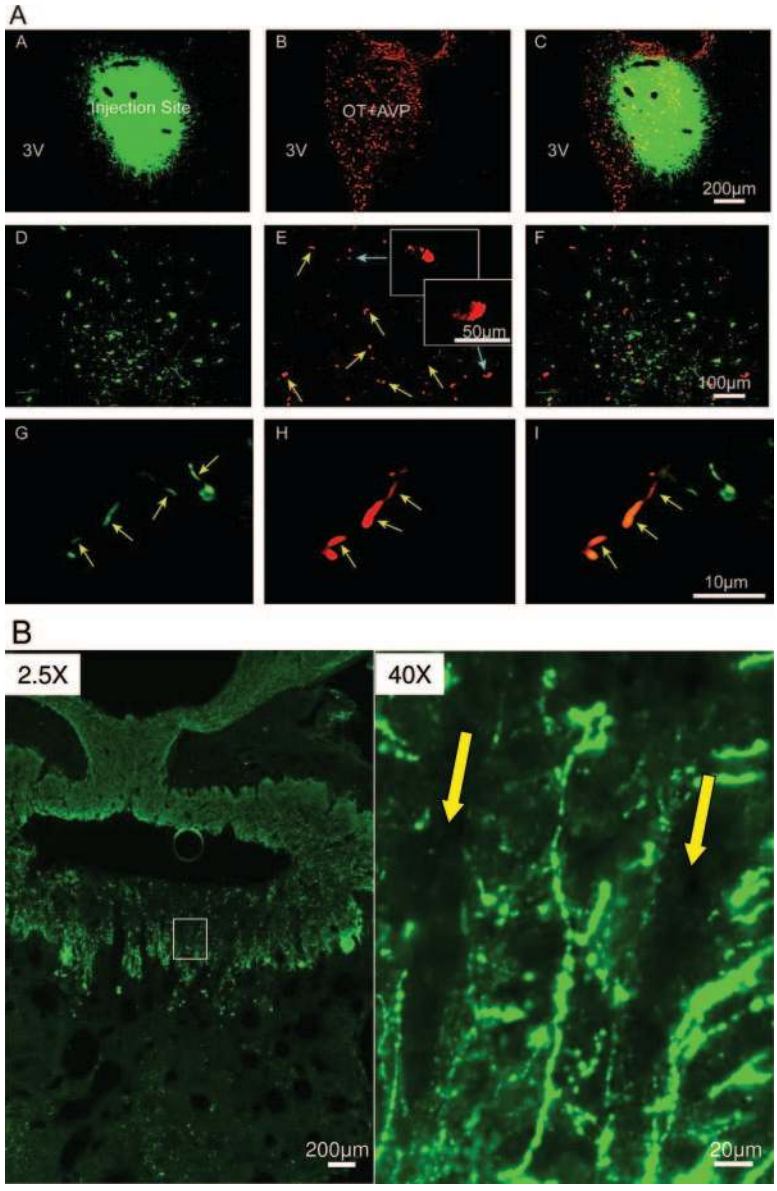

FIG. 4. Panel A, Anterograde tracing of RFRP-3 cells projecting from the PVN to the neurosecretory zone of the median eminence in the ovine brain. A-A, The center of an injection site of BDA in the PVN. A-B, OT/AVP cells in the region of the tracer injection to indicate the location of the injection. A-C, A merged image of A-A and A-B. A-D, An image of the of the injection site of BDA (not in center). A-E, GnIH cells in the same region as A-D. The *insets* in E show immunostained cells indicated by the *blue arrows* at higher magnification, and the *yellow arrows* show other examples of GnIH cells. A-F, A merged image of A-D and A-E. A-G, A BDA positive fiber in the neurosecretory zone of the median eminence. A-H, RFRP-3 staining of the same fiber. A-I, A merged image of A-G and A-H. Panel B, Low-power and high-power images of the neurosecretory zone of the median eminence immunostained with RFRP-3 antiserum, showing labeled fibers in close vicinity to hypophysial portal blood vessels (*arrows*) The *right panel* is a higher power image of the boxed area in the *left panel*. 3V, Third ventricle.

pA_2 value (-log IC_{50}) for the RFRP-3 effect (GraphPad Software Inc., San Diego, CA).

Results

Cloning of ovine RFRP-3 cDNA

The partial sequence obtained by cloning of ovine hypothalamic extract is shown in Fig. 1. Ovine RFRP-1 could be 34 or 12 amino acids, depending on where it is cleaved. Ovine RFRP-3 appears identical to human RFRP-3 (14, 28) (GenBank accession no EU177779), but isolation of mature ovine peptide is required for confirmation.

Identification of RFRP-3 neurons in the ovine brain with projections to the neurosecretory zone of the median eminence

RFRP-3-expressing neuronal cell bodies were found in the DMH and PVN, as well as the region between these two nuclei (Fig. 2). Further indication of the density of these cells is shown by radiolabeling in Fig. 3. Labeling of RFRP-3 expressing cells was not seen in any other region of the ovine brain (data not shown). Anterograde tracing from the PVN showed that cells of this region projected to the neurosecretory zone of the median eminence because RFRP-ir fibers were double labeled with BDA (Fig. 4A) and were found in the vicinity of hypophysial portal blood vessels (Fig. 4B).

Effect of RFRP-3 on LH and FSH release in vitro

Dose-related inhibition of GnRH stimulated LH and FSH secretion was observed when RFRP-3 was applied to cell cultures (Fig. 5, A and B), with no effect of RFRP-3 alone (Fig. 5, C and D). The effect on LH secretion was greater than the effect on FSH secretion. The specificity of effect was confirmed by the lack of effect of de-amidated hamster GnIH. Whereas hamster GnIH had an effect on LH and FSH secretion that was similar to that seen with RFRP-3, the non-amidated hamster peptide had no effect on GnRH-stimulated LH or FSH secretion (Fig. 6, A and B).

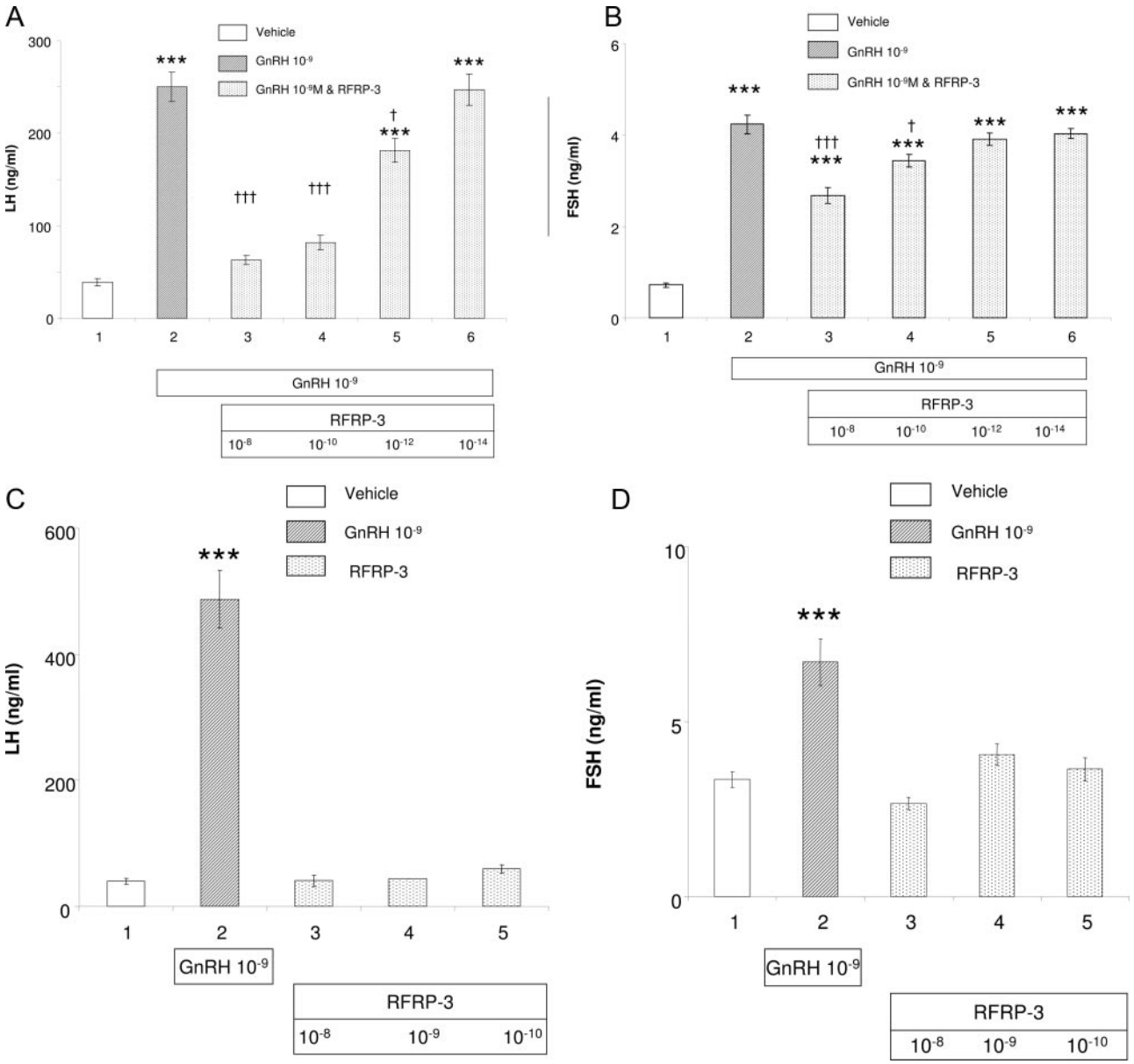

FIG. 5. Inhibitory effect of RFRP-3 on secretion of LH and FSH from ovine pituitary cells in culture. A and B, LH and FSH levels, respectively, in medium of cells receiving vehicle (treatment 1), treated with GnRH (10^{-9} M) or RFRP-3 (10^{-8} to 10^{-14} M), and GnRH combined (treatments 3–7). C and D, LH and FSH secretion in response to RFRP-3 alone. Each data point is the mean (± SEM) of six replicate wells. ***, $P < 0.001$ *vs.* control; †, $P < 0.05$, †††, $P < 0.001$ *vs.* GnRH alone.

Effect of RFRP-3 on LH and FSH release in vivo

Administration (iv) of RFRP-3 to ovariectomized ewes suppressed LH pulse amplitude (control 2.13 ± 0.38 ng/ml, RFRP-3 0.76 ± 0.29 ng/ml; $P < 0.05$) and LH AUC (Fig. 7A) but had no effect on FSH secretion (Fig. 7B). Specificity of action on the gonadotropes was indicated by the absence of effects on either prolactin (Fig. 7C), GH (Fig. 7D), or cortisol (Fig. 7E).

Effect of RFRP-3 on GnRH-stimulated mobilization of intracellular calcium

A standard dose of GnRH increased cytoplasmic calcium in isolated Fluo-4 labeled gonadotropes, and this response was reduced by RFRP3 (Fig. 8A). RFRP-3 reduced this response to GnRH in a dose-dependent manner, with an IC_{50} of 2×10^{-13} M (Fig. 8B).

Discussion

The results of this study provide strong evidence that RFRP-3 is a hypophysiotropic hormone that provides an inhibitory signal from the hypothalamus that acts on the pituitary gonadotropes. Thus, RFRP-3 appears to be a *bona fide* GnIH. This conclusion was reached in the following ways:

1. We identified a cDNA in the ovine hypothalamus that encodes a homolog of RFRP-3. This homolog is identical to that found in human brain.

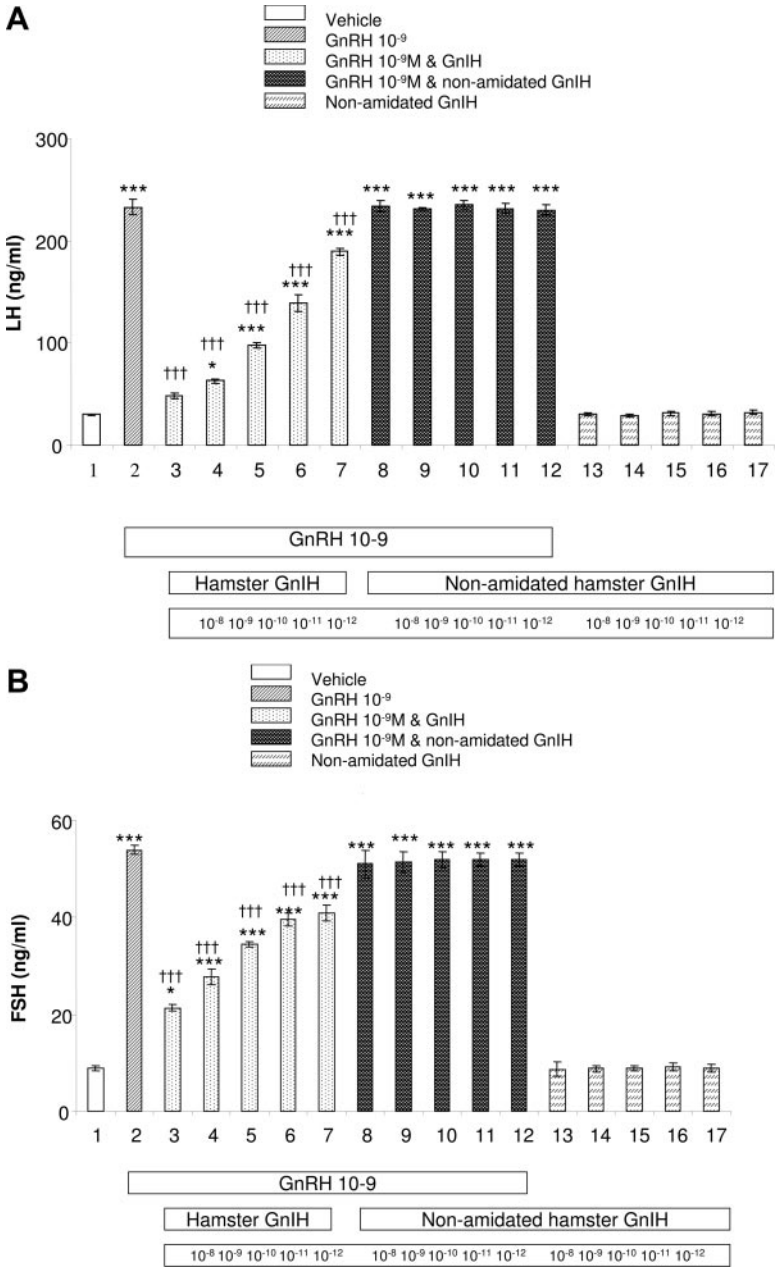

FIG. 6. Lack of an inhibitory effect of non-amidated hamster GnIH on LH and FSH secretion from ovine pituitary cells in culture. A and B, LH and FSH levels, respectively, in medium of cells receiving vehicle (treatment 1), GnRH (10^{-9} M) (treatment 2), GnRH with hamster GnIH (10^{-8} to 10^{-12} M) (treatments 3–7), GnRH with non-amidated GnIH (treatments 8–12), or GnIH alone (treatments 13–17). Each data point is the mean (±SEM) of six replicate wells. *, $P < 0.05$; ***, $P < 0.001$ *vs.* control; †††, $P < 0.001$ *vs.* GnRH alone.

2. RFRP-3 neurons were localized to the PVN and DMH in the ovine brain, and anterograde tracing from the PVN indicated projections of RFRP-3 neurons to the neurosecretory zone of the median eminence.
3. RFRP-3 potently inhibited pituitary gonadotropin secretion from the pituitary gland *in vitro* and when administered iv to ovariectomized ewes *in vivo*.
4. RFRP-3 blocked the calcium signal generated by GnRH in gonadotropes *in vitro*.

The form of RFRP-3 that is found in the ovine brain encodes for a peptide that appears identical to the sequence found in the human brain (15, 29), so it was used in all but one of the experiments in the present study. Ovine/human RFRP-3 differs from the original avian GnIH (SIKPSAYLPLRFamide) that was isolated from quail brain (5), as well as rat (21) and hamster (7) forms of GnIH. These peptides belong to a large family of RF-amide peptides that exert various neuroendocrine effects. For example, prolactin-releasing peptide is a potent releaser of prolactin in rodents (30). Another member of this family that is important in the function of the reproductive axis is kisspeptin, which has a positive influence on GnRH secretion (31). Clearly, the potential exists for the involvement of other RF-amide peptides in neuroendocrine function, making this an emerging field of interest. It is possible that these molecules form a tier of modulators of the well-recognized hypophysiotropic factors, and GnIH seems to oppose specifically the actions of GnRH.

The location of RFRP-3 expressing cells in the ovine brain is within the ventral region of the PVN and throughout the DMH, with cells found between these two nuclei. Whereas the GnIH cells are confined to the PVN in birds (2, 5, 32), the

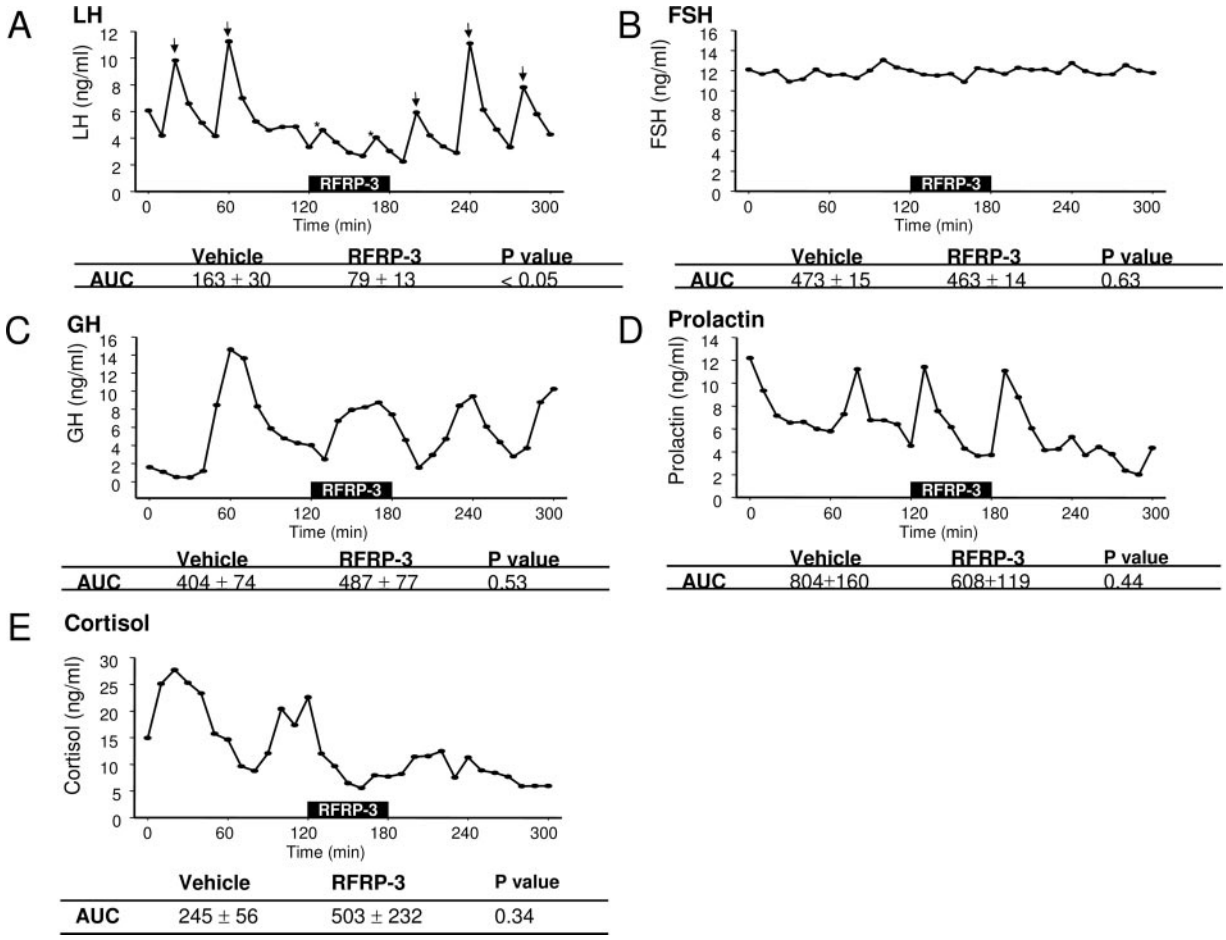

FIG. 7. Inhibitory effect of iv infusion of RFRP-3 on pulsatile LH secretion in ovariectomized ewes. For each panel a representative example is shown for plasma levels of LH (A), FSH (B), GH (C), prolactin (D), and cortisol (E). Mean (± SEM) data for AUC are shown beneath each panel for animals treated with either vehicle or RFRP-3 (n = 3). *Arrows and asterisks* (significantly lower than pretreatment) indicate pulsatile secretory episodes for LH.

location of the neurons is in the DMH of rat, mouse, and hamster brain (7, 33). Our anterograde tracing data indicate that the GnIH (RFRP-3) cells of this region of the ovine brain project to the external zone of the median eminence, which is consistent with the notion that this peptide acts as a hypophysiotropic factor. It now remains to be determined whether RFRP-3 is actually secreted into hypophysial portal blood, and this will require the development of an appropriate assay; such work is in progress. Clearly, the sheep is an ideal species in which to resolve this important issue because hypophysial portal blood sampling is possible (34).

Our *in vitro* data indicate that RFRP-3 is a potent inhibitor of GnRH-stimulated gonadotropin release from gonadotropes, with an effect obtained at picomolar concentrations. Whereas this may appear anomalous with many other physiological systems in which peptides generally act in the nanomolar range, it should be noted that another RF-amide, kisspeptin, is similarly potent in its ability to influence the reproductive axis (35). Our present data obtained with sheep cells add to earlier work in hamsters showing that either intracerebroventricular or ip injection of GnIH reduced plasma LH levels (7). This earlier work did not determine whether GnIH had a central or pituitary site of action. Certainly, there is good evidence in birds, hamsters, rats, and mice that GnIH neurons project to GnRH cells (7, 12, 36), and the peptide may, therefore, act as a neuromodulator, regulating GnRH secretion (2). In addition, avian pituitary expresses GnIH receptor (11, 12), and there is some evidence in birds for a role for GnIH in the regulation of gonadotropin secretion by direct action on the pituitary gland, but definitive proof of a specific effect in mammals has not been forthcoming until now. We present data to show that RFRP-3 potently inhibits gonadotropin secretion in the sheep, and the effect is specific to the gonadotrope. First, a non-amidated preparation of hamster GnIH had no effect on pituitary cells, whereas amidated hamster GnIH inhibited gonadotropin secretion to the same extent as RFRP-3. Second, with *in vivo* administration, an effect was seen only on LH secretion, and not on plasma levels of prolactin, GH, or cortisol. This demonstration of specificity is important because there are reports of an effect of kisspeptin and RFRP-3 to stimulate the GH axis in cattle (6, 37), so specificity of RF-amide peptide action becomes an issue. Although we did not examine an effect on the thyroid axis, it appears that the effect of GnIH is specific to the pituitary gonadotropes. It

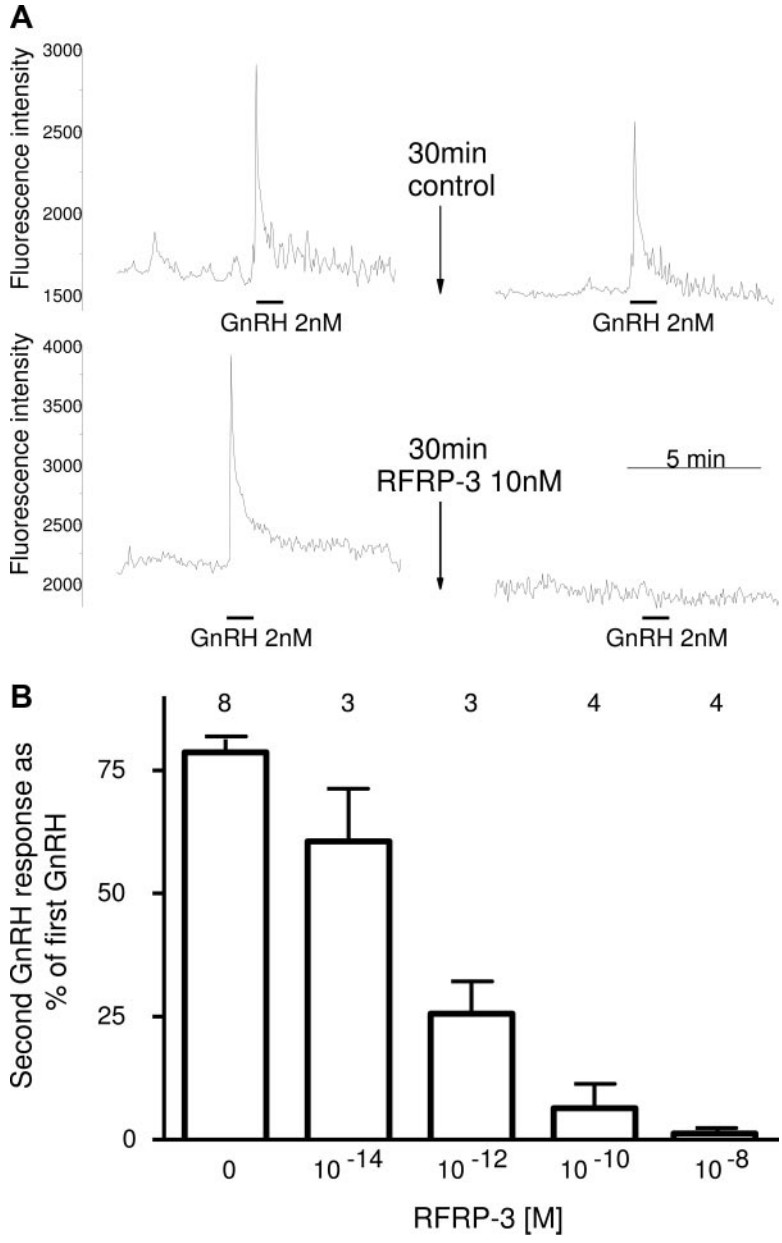

FIG. 8. Inhibitory effects of RFRP-3 on cytoplasmic Ca^{2+} mobilization in ovine pituitary gonadotropes, as measured by the intensity of Fluo-4 fluorescence, upon stimulation with GnRH. A, Representative examples of the calcium signaling in gonadotropes stimulated with 2 nM GnRH at 30-min intervals, with either vehicle (*top panel*) or RFRP-3 (10 nM) (*bottom panel*) treatment between the two stimuli. B, Mean (± SEM) responses (recordings from three to eight sheep) in cells treated with 10^{-8} to 10^{-14} M RFRP-3. The numbers across the graph in B represent the number of sheep from which cells were sourced, and 10–30 cells were recorded per sheep.

will be informative to determine the location of the GPR147, the putative GnIH receptor (38).

Our *in vitro* experiments showed an effect of RFRP-3 treatment on GnRH-stimulated FSH secretion, but there was no effect of a 1-h infusion on FSH levels in ovariectomized ewes *in vivo*. Notably, the *in vitro* effect on FSH secretion was less than that on LH secretion, and this may be due to the difference in the mechanism of control of secretion of the two gonadotropins. In addition, an *in vivo* effect of GnIH upon LH levels was shown in quail, with reduction in α-subunit and LHβ mRNA in the pituitary gland, but not on FSHβ subunit or plasma FSH levels (39). That study did not determine a direct pituitary effect, although another study using avian pituitary cultures showed that GnIH reduced FSH and LH secretion with an effect on α-subunit and FSHβ expression, and not LHβ (9). Whereas pulsatile LH secretion is wholly dependent upon secretagogue actions of GnRH, this is not the case for FSH secretion (40), and apparent pulsatile secretion of FSH is not tightly coupled to GnRH input (40, 41). Accordingly, one would expect that the relatively lower FSH secretory response to GnRH that is seen *in vitro* would be less influenced by a suppressive factor than would the LH response. It remains possible that more prolonged treatment with RFRP-3 negatively regulates FSH secretion.

We found that GnIH alone had no effect on the secretion of gonadotropins from ovine cells in culture, demonstrating that there is no singular action of the peptide on these cells. This is in accord with the well-known fact that gonadotropes require the tropic stimulus of GnRH to function. In the absence of pulsatile GnRH input, gonadotropes neither produce nor secrete gonadotropins (42, 43).

Our data unequivocally show that RFRP-3 potently blocks the generation of intracellular free calcium elicited by GnRH. Similar to the finding on gonadotropin secretion, RFRP-3 alone was without effect on basal cytoplasmic calcium levels (data not shown). An increase in cytoplasmic calcium levels is essential for the secretion of LH by GnRH in these cells (44). The generation of inositol trisphosphate is responsible for the initial large increase in cytoplasmic calcium (45) and LH release in response to GnRH (46, 47), and it is this component of the response that is reduced by RFRP-3. Studies are in progress to probe the site(s) of RFRP-3 action within this second messenger system. Further studies are also in progress to determine whether RFRP-3 acts on other intracellular pathways and the extent to which it may affect the expression of gonadotropin subunits.

In summary, we present data that indicate a role for GnIH as a hypophysiotropic hormone. A gene encoding RFRP-3 is identified in the ovine hypothalamus, and the cells are localized to the PVN/DMH. These cells project to the neurosecretory zone of the median eminence, and RFRP-3 exerts potent and specific effects on pituitary gonadotropes. Accordingly, the secretory response of these cells is profoundly suppressed by RFRP-3, through a negative influence on the generation of intracellular free calcium.

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