Unsaturated Fatty Acids Stimulate LH Secretion via Novel PKCε and -θ in Gonadotrope Cells and Inhibit GnRH-Induced LH Release

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The activity of pituitary gonadotrope cells, crucial for reproductive function, is regulated by numerous factors including signals related to nutritional status. In this work, we demonstrated, for the first time, that *in vivo* central exposure of rats to lipids intracarotid infusion of a heparinized triglyceride emulsion selectively increases the expression of pituitary LH subunit genes without any alteration of pituitary GnRH receptor and hypothalamic GnRH or Kiss-1 transcript levels. Furthermore, we showed that unsaturated fatty acids (UFA), oleate and linoleate, increase LH release in a dose-dependent manner as well as LHβ mRNA levels in both immortalized LβT2 gonadotrope cell line and rat primary cell cultures. In contrast, the saturated palmitate was ineffective. ACTH or TSH secretion was unaffected by UFA treatment. We demonstrated in LβT2 cells that linoleate effect is mediated neither by activation of membrane fatty acid (FA) receptors GPR40 or GPR120 although we characterized these receptors in LβT2 cells, nor through nuclear peroxisome proliferator-activated receptors. Furthermore, linoleate β-oxidation is not required for its action on LH secretion. In contrast, pharmacological inhibition of protein kinase C (PKC) or ERK pathways significantly prevented linoleate-stimulated LH release. Accordingly, linoleate was shown to activate novel PKC isoforms, PKCε and -θ, as well as ERK1/2 in LβT2 cells. Lastly, unsaturated, but not saturated, FA inhibited GnRH-induced LH secretion in LβT2 cells as well as in pituitary cell cultures. Altogether, these results suggest that the pituitary is a relevant site of FA action and that UFA may influence reproduction by directly interfering with basal and GnRH-dependent gonadotrope activity. **(*Endocrinology* 152: 3905–3916, 2011)**

Gonadotropin hormones, LH and FSH, released by pituitary gonadotrope cells, are the main hormones regulating gonadal activity. Indeed, LH and FSH act in a coordinated manner on gonads to promote both gametogenesis and steroidogenesis. The careful regulation of gonadotropin hormones is vital to full reproductive function and is achieved by a complex combination of hypothalamic peptides and endocrine or paracrine signals (1, 2). Among these factors, the hypothalamic neurohormone GnRH is the main stimulatory signal eliciting gonadotropin synthesis and release. Binding of GnRH to its receptor of the G protein-coupled receptor (GPCR) family initiates a wide array of signaling events, mainly through the initial recruitment of phospholipase Cβ (3, 4). GnRH mobilization of intracellular Ca^{2+}, together with Ca^{2+} influx, regulates acute gonadotropin release. In addition to triggering gonadotropin exocytosis, elevation of intracellular Ca^{2+} levels activates the nitric oxide synthase type I (NOS1) cascade (NOS1/NO/soluble guanylate cyclase) (5). GnRH activates several protein kinase C (PKC) isoforms that play a pivotal role in mediating the subsequent activation of different signaling cascades. Indeed, PKC activate the phospholipases D and A2 as well as the MAPK cascades. Furthermore, novel PKCδ and -ε functionally

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Abbreviations: FA, Fatty acid; GnRHa, GnRH agonist; GnRH-R, GnRH receptor; GPCR or GPR, G protein-coupled receptor; NOS I, nitric oxide synthase type 1; P-ERK, phosphorylated ERK; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; UFA, unsaturated FA.

couple the GnRH receptor (GnRH-R) to the cAMP pathway (6). PKC, cAMP, and ERK pathways all contribute to GnRH-stimulated transcription of gonadotropin genes (7).

In addition to GnRH, the secretion of gonadotropin hormones depends on other hypothalamic peptides such as pituitary adenylyl cyclase-activating polypeptide or gonadal-derived steroid and peptides hormones (8, 9). Furthermore, the activity of pituitary gonadotrope cells can also be regulated by factors linked to nutritional status. Indeed, it has been reported for a long time that metabolic perturbations associated with under- or overnutrition affect gonadotropin release in numerous species of mammals including humans (10, 11). Several metabolic signals mediating nutritional regulation of reproduction have been identified including insulin, adipokines, or enterokins (12–14). Part of the effects exerted by these nutritional signals can be explained by their central action on the activity of hypothalamic GnRH neurons and hence on GnRH secretion. However, this does not exclude a direct action on pituitary gonadotrope cells. There is indeed a growing amount of data reporting that insulin, leptin, or the more recently discovered adipokine, adiponectin, can interfere with basal or GnRH-induced LH release by pituitary gonadotrope cells (15–20).

Reproductive activity can also be directly influenced by nutrients. Glucose is an important metabolic regulator of reproductive function, and glucodeprivation has been reported to decrease LH secretion in several species (21). Glucose can be sensed by hypothalamic neurons, and central gluco-sensing contributes to the observed effects of glucodeprivation on LH secretion (22). Interestingly, there is increasing evidence that in addition to glucose, circulating fatty acids (FA) can also directly act into the brain to regulate food intake and energy homeostasis (23–25), suggesting that FA signaling may interfere with the neuroendocrine control of reproduction. However, despite the growing amount of data obtained notably in domestic ruminant animals (26) indicating that FA-enriched diets interfere with reproductive processes, the potential underlying mechanisms are still poorly understood. The mechanisms by which free FA and/or their derivatives exert their action in several peripheral tissues, including pancreas, liver, adipose tissue, and skeletal muscle, have been extensively studied. FA take part in cellular processes by providing an oxidative energy source. They can also regulate gene transcription, notably via activation of nuclear receptors of the peroxisome proliferator-activated receptor (PPAR) family or other transcription factors (27, 28). More recently, it has been demonstrated that FA can act as ligands of membrane receptors belonging to the GPCR family (29). In addition, the regulatory effects of FA occur by their involvement in intracellular lipid-signaling pathways. FA can notably activate, directly or indirectly via diacylglycerol production, several PKC isoforms, and this has been involved in FA-induced peripheral insulin resistance (30). FA can also activate kinases including ERK, phosphatidylinositol 3-kinase (PI3K), or AMP-activated kinase and hence modulate cellular signaling pathways (31).

The present study was undertaken to assess whether FA can interfere with the hypothalamo-pituitary control of reproduction and to identify some of the underlying mechanisms. Using a rat model of central lipid overload, we reported *in vivo* stimulatory effects of lipids on pituitary LH subunit gene expression. Furthermore, using both rat pituitary cell cultures and the murine gonadotrope cell line LβT2, we demonstrated a specific stimulatory action of unsaturated FA (UFA) on LH release and LHβ transcripts levels. Lastly, we provided evidence that this effect is mediated, at least in part, via UFA activation of novel PKC isoforms and ERK.

Materials and Methods

Materials

FA, oleate, linoleate, and palmitate; pharmacological inhibitors, GW9508, Etomoxir, GF109203X, U0126, GW7647, GW9662; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; the GnRH agonist (GnRHa) [D-Trp (6)]GnRH as well as PKCα (4334) and PKCε (8458) antibodies were from Sigma (Saint-Quentin Fallavier, France). GW1100 was kindly donated by GlaxoSmithKline (Brentford, UK). Antibodies against phosphorylated ERK (P-ERK)1/2, total ERK, and phospho-PKCδ (Thr505) were from Cell Signaling (Ozyme, France). Antibodies against PKCθ (sc 212) and PKCζ (sc 216) were from Santa Cruz Biotechnology (Tebu, France).

Carotid infusion of lipids

Animals were housed and maintained according to the Institutional Animal Care and Use Committee of Paris 7 University (Agreement A75–13-17, Centre National de la Recherche Scientifique, Paris 7 University, Paris, France). The experimental protocol was approved by the local Ethical Committee (Agreement CEEA40). We have used a rat model allowing a direct central FA delivery, as previously described (32). Briefly, adult male rats, housed at 24 ± 3 C with a 12-h light, 12-h dark cycle, were anesthetized ip with pentobarbital (50 mg/kg of body weight; Sanofi, Paris, France) for surgical placement of a catheter in the right carotid artery toward the brain. After 5 d, animals received a continuous 24-h infusion of vehicle (NaCl 0.9%) or triglyceride emulsion (Intralipid 20%; KateVitrum, Sweden) beginning at 0010 h, both containing heparin (20 U/ml), at a rate of 2 μl/min. Heparin was added to the lipid emulsion to stimulate lipoprotein lipase activity and thus to release FA. The major FA of Intralipid are polyunsaturated linoleate and linolenate (~61%), monounsaturated oleate (~ 23%), and saturated palmitate and stearate (~16%). Rats were killed by decapita-

tion, and anterior pituitary gland and hypothalamus were rapidly excised. The hypothalamus was dissected within the well demarcated limits with micro-scissors curved, and the depth of dissection was approximately 3 mm. Organs were deep frozen in liquid nitrogen whereas sera were stored at −20 C. Plasma FA concentrations were determined according to the manufacturer's specifications (NEFA-C test; Wako Chemicals GmbH, Neuss, Germany).

LβT2 cells culture

Pituitary gonadotrope cell line LβT2 generated by Pamela Mellon (33, 34) were grown in DMEM (LONZA, Braine-l'Alleud, Belgium) supplemented with 4 mM glutamine, 10% fetal calf serum (PANbiotech; Dutscher, France) and 50 μg/ml gentamycin at 37 C in a humidified atmosphere with 5% CO_2. LβT2 cells (1×10^6 cells per well) were grown to 80% confluence in 12-well plates, starved overnight in serum-free DMEM, and incubated for up to 4 h with FA (10–200 μM). FA were either methyl-β-cyclodextrin- (linoleate and oleate) or BSA-complexed forms (ratio BSA to palmitate, 1:5). The effects of FA treatment were evaluated on LH release, LH mRNA levels, Ca^{2+} mobilization, or PKC and ERK activation. For measurement of FA effect on basal or GnRH-induced LH secretion, cells were extensively washed and further incubated for a next 4-h period with or without GnRHa as detailed in the text of the respective figure legends. When tested, pharmacological inhibitors GW9662 (20 μM), GF109203X (4 μM), U0126 (10 μM), Etomoxir (100 μM), or GW1100 (10 μM) were added 1 h before FA addition. Cells were also stimulated with the GPR40/120 agonist GW9508 (10 μM). At the end of each experiment, medium was stored at −20 C for determination of LH release. All the experiments were performed in triplicate.

Primary culture of anterior pituitary cells

Anterior pituitary glands were removed from adult female Wistar rats (225–250 g, Janvier, CERJ, Le Genest Saint Isle, France) and cells were enzymatically dispersed using the trypsin dissociation procedure, as previously described (35). After overnight incubation in serum-free Ham F-10, cells (1×10^6) were treated for 4 h with FA (10–200 μM), and mRNA levels or LH release was measured as described for LβT2 cells.

RNA analysis by real-time RT-PCR

Total RNA from LβT2 and cultured pituitary cells and from rat anterior pituitary gland or hypothalamus were isolated with an RNeasy-kit (QIAGEN, Courtaboeuf, France). First-strand cDNA was obtained from 1 μg RNA with Superscript II reverse transcriptase (Invitrogen, France) according to the manufacturer's instructions. For characterization of GPR40 and 120 gene expression, classical PCR amplifications were performed using a Bio-Rad C1000 thermal cycler. For *Lhb*, *Nos1*, *Gnrh-r*, *Cga*, *Kiss1*, and *Gnrh* mRNA level quantifications, real-time PCR was carried out in the LightCycler 480 Instrument (Roche Diagnostics, Meylan, France) using 6 μl of LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics), 5 μl of a 1:25 to 1:60 cDNA dilution, and 0.5 μM of each primer. The oligonucleotide primer sequences used are indicated in Table 1. PCR product was identified as a single band on ethidium bromide gel. Expression levels were normalized to *Cyclophilin*, used as an internal control. Data were analyzed using the advanced-E-method with standard curve-derived efficiencies obtained from LightCycler 480 software.

Calcium mobilization assay in LβT2 cells

LβT2 cells were plated (150,000 cells per well) into poly-D-lysine-coated 96-well clear-bottomed black microplates (Greiner Bio-one, Dutscher) 24 h before assay. Cells were incubated or not with FA or methyl-β-cyclodextrin for 4 h, and then extensively washed and Ca^{2+} dye loaded (FLIPR calcium 5 assay kit; Molecular Devices, Sunnyvale, CA) for 1 h before stimulation with GnRHa. For measurement of immediate FA effect, cells were Ca^{2+} dye loaded for 1 h and stimulated with linoleate. Excitation fluorescence was 485 nm, and emission was detected at 525 nm using a 515-nm cutoff filter. Fluorescent signals were recorded every 1.5 sec for 4 min. The profiles shown are representative of three independent experiments, and each indicated value is an average from three wells.

Protein extraction and immunoblotting

For whole-cell extract preparation (ERK detection), LβT2 cells were washed after FA treatment with ice-cold PBS supplemented with 10 mM NaF and 100 μM Na_3VO_4 and homogenized in a buffer containing 10 mM Tris-HCl (pH 7.4), 30 mM NaPPi, 50 mM NaCl, 1% TritonX-100, and 1 mM dithiothreitol supplemented with protease and phosphatase inhibitor cocktails (Roche Diagnostics). Homogenates were centrifuged at 20,000 × *g* for 30 min at 4 C.

For cytosolic and membrane fraction preparation (PKC detection), LβT2 cells were scraped after FA treatment into homogenization buffer [25 mM Tris-HCl (pH 7.5) containing 2 mM EDTA, 2 mM EGTA supplemented with 2 mM dithiothreitol, and protease and phosphatase inhibitor cocktails]. Cells were then disrupted with Dounce homogenizer and the homogenates cen-

TABLE 1. Oligonucleotide primer sequences used for PCR and real-time PCR

Target cDNA	Amplicon size	Forward primer	Reverse primer
Lhb	229 bp	5′-ATCACCTTCACCACCAGCAT-3′	5′-GACCCCCACAGTCAGAGCTA-3′
Cga	159 bp	5′-GCTGTCATTCTGGTCATGCT-3′	5′-GAAGCAACAGCCCATACACT-3′
Gnrh-r	246 bp	5′-TCAGCTGCCTGTTCATCATC-3′	5′-AACATTTCCGGATCAAACCA-5′
Nos1	123 bp	5′-TGCAGGAGGAGAGAAAGAGC-3′	5′-CCAGGGGTCCAGTACTTTCA-3′
Gnrh	168 bp	5′-CCAGCACTGGTCCTATGGGT-3′	5′-AGAGCTCCTCGCAGATCCCT-3′
Kiss1	150 bp	5′-CTGCTGCTTCTCCTCTGTGT-3′	5′-AGGCTTGCTCTCTGCATACC-3′
Gpr40 (Ffar1)	139 bp	5′-GCTATTCCTGGGGTGTGTGT-3′	5′-GAGCCATTCACGGGTATGTT-3′
Gpr120	104 bp	5′-AGAGGCTTACGCTGAGCTTG-3′	5′-GAAGGAAACCATGAGCAGGA-3′
Cyclophilin	109 bp	5′-CAAAGTTCCAAAGACAGCAG-3′	5′-CTGGCACATGAATCCTGGAA-3′

Primer pairs were designed to target cDNA fragments encompassing at least one intron in the gene sequence to prevent amplification of genomic DNA.

trifuged at 48,000 × *g* for 1 h at 4 C. The supernatant (cytosol fraction) was collected and the pellet was resuspended in cold homogenization buffer containing 1% Triton-X100, sonicated, and centrifuged at 48,000 × *g* for 30 min. The resulting supernatant corresponded to the membrane fraction.

Protein extracts (15–30 μg) were separated on a 10% SDS-PAGE. After electrotransfer onto nitrocellulose membrane, P-ERK1/2, PKCα, -ε, -θ, -ζ or phospho-δ were immunodetected using specific antibodies and enhanced chemiluminescent detection system (ECL+, GE Healthcare Europe Gmbh, Orsay, France). Blots were analyzed with a Fuji LAS-4000 imager and quantified with a MultiGauje software analysis. Total ERK and β-actin were used as internal loading control for P-ERK and PKC expression, respectively.

Pituitary hormones assay

In cell culture media, LH concentration was measured using an ELISA method (adapted from Ref. 36) with reagents supplied by Dr. Parlow (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA). Briefly, microtitration plates (High binding, Greiner Bio-one) were coated overnight at 4 C with 10 ng of purified rat LH-I-10 diluted in carbonate buffer. Rat LH-RP3, used as standard, and media were incubated with antirat LH-S11 (dilution 1:4000 overnight at 4 C). Plates were then blocked with PBS containing 1% BSA and 0.1% Tween 20 for 1 h at room temperature and washed with PBS-0.1% Tween 20 before addition of standards and samples for competition binding (2 h at 4 C). After removal of unbound material, phosphatase alkaline-labeled secondary antibody (dilution 1: 2000, Thermo Scientific, Illkirch, France) was added for 1 h at 4 C, and phosphatase alkaline activity was revealed with SigmaFast pNPP reagent. The minimum detectable LH concentration was 0.2 ng/ml, and the interassay coefficient of variation was less than 10%. ACTH and TSH concentrations in culture media and serum LH concentration were determined with commercial kits (ImmunoDiagnostic Systems, Pouilly en Auxois, France).

Statistical analysis

For *in vivo* as well as *in vitro* studies, all given values are the mean ± SEM of at least three independent experiments. Three to five control or Intralipid-treated rats were used in each experiment. Experiments using pituitary or LβT2 cell cultures were performed in triplicate except for Western blot, which were done in duplicate. Statistical differences were first determined by one-way ANOVA followed by Dunnett's *t* test for multiple comparisons. Individual pair-wise comparisons were performed using Student's *t* test. *, $P \leq 0.05$ was considered significant.

Results

In vivo central infusion of lipids increases pituitary transcript levels of LH subunits

To determine whether FA could directly regulate gonadotrope activity *in vivo*, we selectively increased central FA levels by intracarotid infusion of rats with a triglyceride emulsion in the presence of heparin to stimulate lipoprotein lipase activity. This treatment did not significantly modify systemic plasma FA concentrations (431 ± 34 and 336 ± 38 mM in infused and control rats, respectively; data not shown). Central lipid infusion significantly increased the levels of pituitary *Cga* and *Lhb* mRNA (Fig. 1A). In contrast, transcript levels of other important factors of pituitary gonadotrope activity such as the GnRH-R or NOS I enzyme, known to be up-regulated by GnRH, were not affected. Because the observed increase in LH subunit gene expression could be consecutive to a hypothalamic action of FA on the gonadotrope drive, we evaluated hypothalamic levels of transcripts coding for GnRH or for

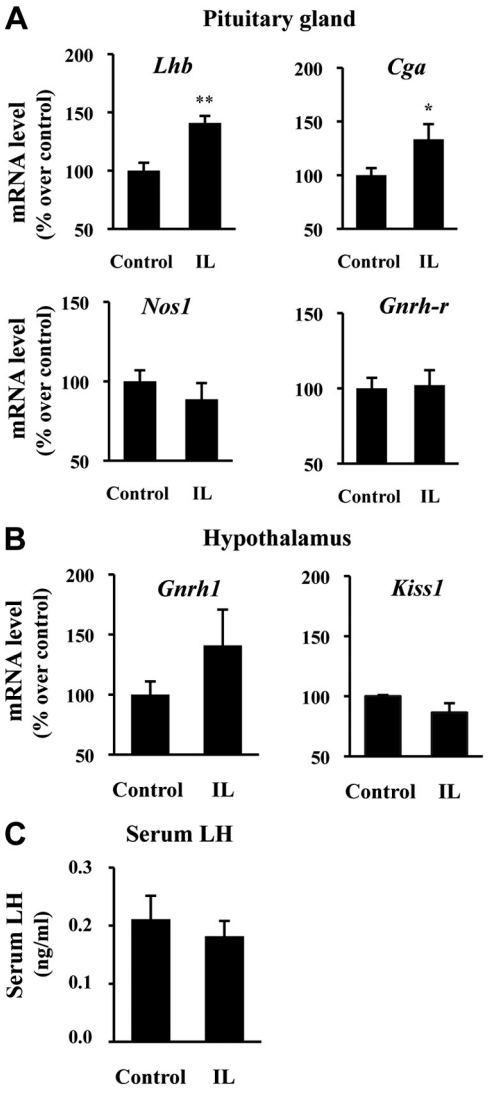

FIG. 1. Central elevation of lipids up-regulates the expression of anterior pituitary *Lhb* and *Cga* genes. Adult rats received a 24-h carotid-artery infusion (2 μl/min) of heparinized NaCl 0.9% (Control) or 20% Intralipid emulsion (IL). Heparin was added to stimulate lipoprotein lipase activity. The levels of several mRNA were measured in pituitary gland (A) and hypothalamus (B) by real-time RT-PCR, and the serum LH concentration (C) was determined as described in *Materials and Methods.* Results are expressed as percentage over control group and are the mean ± SEM of three independent experiments with three to five control or Intralipid-treated rats in each experiment. *, $P \leq 0.05$; **, $P \leq 0.01$ compared with control group.

Kiss-1, the main activator of GnRH neuron activity. No significant increase of *Gnrh* or *Kiss1* transcripts was induced by central lipid infusion (Fig. 1B) nor did treatment significantly modify serum LH concentrations (Fig. 1C).

Unsaturated oleate and linoleate, but not the saturated palmitate, promote LH release and increase LHβ transcript levels in primary cultures of pituitary cells and LβT2 gonadotrope cell line

To determine whether unsaturated or saturated FA may directly act on gonadotrope cells, we have treated LβT2 cells with increasing FA concentrations for 4 h and measured LH release during the next 4 h-period. As shown in Fig. 2A, unsaturated linoleate or oleate induced a dose-dependent increase in LH secretion with a maximal effect at 200 μM. Coincubation of cells with both UFA did not further enhance LH release (1.6-fold increase over basal; data not shown). In contrast, the saturated palmitate did not affect LH secretion (Fig. 2A), and increasing its concentration up to 400 μM still did not influence LH release (data not shown). Because unsaturated fatty acids used were methyl-β-cyclodextrin encapsulated, we evaluated the effects of methyl-β-cyclodextrin alone on LH secretion. No modification of LH release was detected, even with maximal concentrations of methyl-β-cyclodextrin (Fig. 2A), indicating that the observed effects of UFA are specific. The effects of UFA on LH release were also determined in primary cultures of rat anterior pituitary cells (Fig. 2B). As observed in the LβT2 gonadotrope cell line, treatment with UFA significantly enhanced subsequent LH secretion in a dose-dependent manner. Maximal increase (1.9-fold) was obtained at 200 μM for both UFA. Again, as observed in LβT2 cells, treatment with palmitate or methyl-β-cyclodextrin alone was ineffective. To determine whether UFA could affect other pituitary endocrine cells, we evaluated the effects of linoleate on ACTH and TSH release. No significant change could be observed for either hormone (106 ± 4 and 105 ± 7% over basal for ACTH and TSH, respectively; data not shown), suggesting that receptivity to UFA is restricted to some but not all endocrine cells of the pituitary. Evaluation of cell viability (Table 2) revealed that treatment with UFA concentrations up to 200 μM had no effect on viability of LβT2 cells. At 200 μM, UFA reduced viable cell numbers in primary cultures. In both cell types, viability was not affected by palmitate or methyl-β-cyclodextrin.

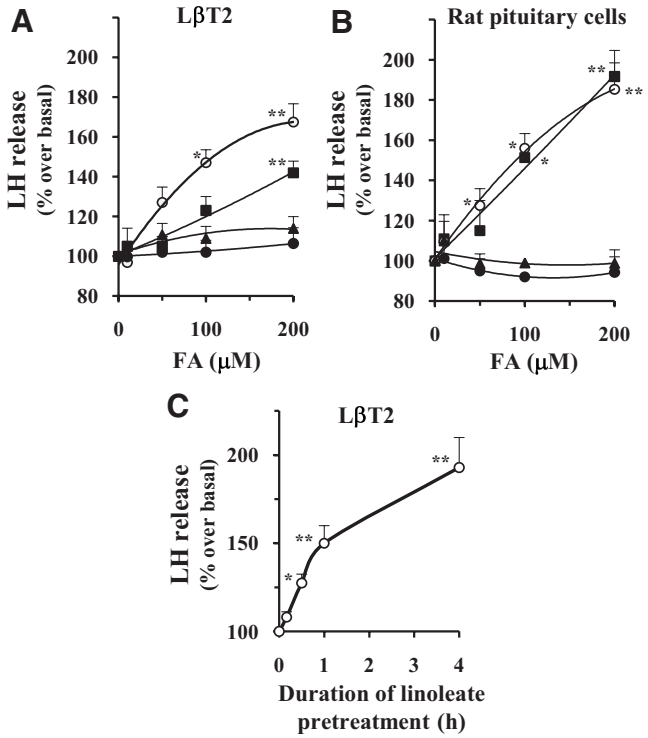

FIG. 2. UFA enhance the secretory activity of gonadotrope cells. Effects of a 4-h treatment with increasing concentrations (0–200 μM) of linoleate, oleate, or palmitate on LH release in LβT2 gonadotrope cells (panel A) and primary cultures of rat anterior pituitary cells (panel B). Cells were incubated or not with unsaturated linoleate (○), oleate (■) or saturated palmitate (▲) for 4 h. Cells were also treated with methyl-β-cyclodextrin (●) used to encapsulate unsaturated FA. Concentrations of methyl-β-cyclodextrin were 0.12, 0.25, 0.5, and 1 mM for 10, 50, 100, and 200 μM of UFA, respectively. Cells were then extensively washed and LH release was assayed by ELISA after an additional 4-h period, as described in *Materials and Methods*. C, Time course of linoleate effect on LH release in LβT2 cells. Cells were incubated with 200 μM linoleate for 15 min, 30 min, 1 h, or 4 h, and LH release was measured after an additional 4-h period. Results are expressed as percentage over basal and are the mean ± SEM of three to eight experiments. Basal LH release was in the range of 30–50 and 2–5 ng/ml/10^6 cells/4 h in rat pituitary and LβT2 cells, respectively. *, $P \leq 0.05$; **, $P \leq 0.01$ compared with basal.

In a first attempt to identify the mechanisms by which UFA interfere with LH release, we evaluated the time-course of UFA effect on LH secretion. This was done by incubating LβT2 cells for increasing periods of time (15 min to 4 h) with linoleate (200 μM) and measuring LH release after a next 4-h incubation period. As illustrated in Fig. 2C, treatment with linoleate led to a progressive time-dependent increase in subsequent LH release. A weak effect of UFA could be detected after a 30-min pretreatment and was maximal at 4 h, indicating that the maximal stimulatory effect of UFA on gonadotrope cells is not immediately operative.

Because the delayed effects of UFA on gonadotrope cells suggest that they may require neosynthesis of LH, we next determined the consequences of UFA treatment on LH subunit mRNA levels. Levels of transcripts coding for the glycoprotein α and LHβ subunits were measured by real-time RT-PCR in rat pituitary and LβT2 cells after a 4-h treatment with UFA (Fig. 3). In both types of cells, we found that UFA, linoleate, and oleate, significantly increased *Lhb* transcript levels (153 ± 5

TABLE 2. Effects of a 4-h treatment with linoleate, oleate, or palmitate on the viability of rat pituitary cells and LβT2 gonadatrope cells

	Rat anterior pituitary cells (% over control)				LβT2 cells (% over control)
FA concentration (μM)	10	50	100	200	200
Linoleate	105 ± 7	115 ± 15	88 ± 10	61 ± 5*	98 ± 11
Oleate	97 ± 10	129 ± 20	82 ± 11	48 ± 4*	102 ± 10
Palmitate	93 ± 5	95 ± 10	ND	112 ± 20	100 ± 12
Methyl-β-cyclodextrin	98 ± 11	95 ± 12	ND	122 ± 16	104 ± 6

The final concentrations of methyl-β-cyclodextrin were 0.125, 0.25, 0.5, and 1 mM, respectively, for 10, 50, 100, and 200 μM of FA. All values are expressed relative to control untreated cells and are the mean ± SEM of three independent experiments. *, $P \leq 0.05$, compared with control cells.

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After FA treatment, medium was renewed and cells incubated for 4 h in a medium containing 1 mg/ml MTT. Medium was then discarded and the purple crystal granules were dissolved in dimethylsulfoxide. The absorbance of solubilized formazan crystals was measured at 575 nm. ND, Not determined.

and 142 ± 6% over basal in response to 200 μM linoleate and 145 ± 18 and 150 ± 5.5% over basal in response to 200 μM oleate, in LβT2 and pituitary cells, respectively). Treatment of rat pituitary or LβT2 cells with either methyl-β-cyclodextrin or palmitate was ineffective. No effect was observed on *Cga* transcript levels with any of the FA tested (Fig. 3).

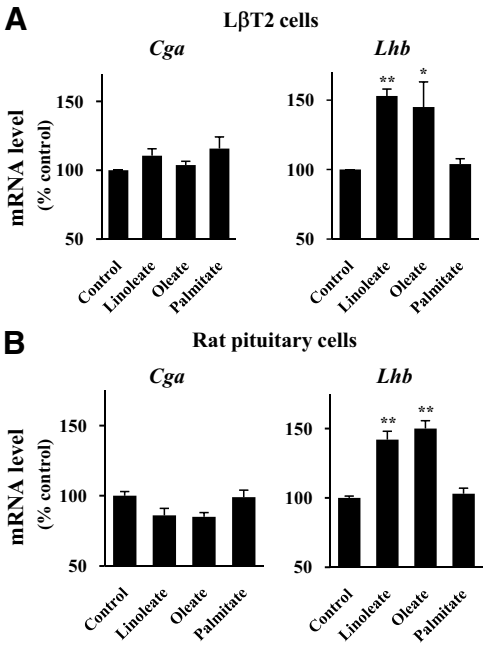

FIG. 3. UFA stimulate *Lhb* gene expression in gonadotrope cells. Effects of linoleate, oleate, or palmitate on *Cga* and *Lhb* mRNA levels in LβT2 cells (panel A) and primary cultures of rat anterior pituitary cells (panel B). Cells were incubated with 200 μM linoleate, oleate, or palmitate for 4 h. Total RNA was extracted, and *Cga* and *Lhb* mRNA levels were determined by real-time RT-PCR. The cycling conditions included an initial heat-denaturing step at 95 C for 10 min, 40 cycles at 95 C for 10 sec, annealing at 60 C for 10 sec, and product elongation and signal acquisition at 72 C for 10 sec, followed by melting curves determination. Levels of *Cga* and *Lhb* mRNA were not modified by treatment with 1 mM methyl-β-cyclodextrin alone. Results are expressed as percentage over control and are the mean ± SEM of four to six experiments. *, $P \leq 0.05$; **, $P \leq 0.01$ compared with control cells.

Linoleate-induced LH release in LβT2 cells is mediated through activation of novel PKC and ERK1/2

Several studies have reported that medium- to long-chain FA can signal directly via membrane receptors GPR40, also known as FFAR1, and GPR120, both belonging to the GPCR family (29). We thus examined by RT-PCR whether GPR40 and GPR120 are expressed in gonadotrope cells and rat anterior pituitary gland. *Gpr40* transcripts were not detected in either rat anterior pituitary gland or primary cultures of rat pituitary cells, whereas mouse LβT2 cells expressed them (Fig. 4A). In contrast, we found significant levels of *Gpr120* transcripts in both rat pituitary and LβT2 cells. Because GPR40 and 120 are coupled to phospholipase C, we first evaluated the ability of linoleate to increase intracellular calcium in LβT2 cells using a fluorometric imaging plate reader. As shown in Fig. 4B, whereas stimulation of cells with GnRHa (10 nM) caused a rapid and robust increase in Ca^{2+} levels, linoleate had no effect. To determine the potential contribution of GPR-activated pathways in the stimulation of LH release by UFA, we next stimulated LβT2 cells with the GPR40/120 agonist GW9508 (10 μM). GW9508 treatment was not able to mimic the effect of UFA on LH release (Fig. 4C). Furthermore, incubation of cells with linoleate (200 μM) in the presence of the GPR40/120 antagonist GW1100 (10 μM) did not prevent the stimulatory effect of linoleate (Fig. 4C), clearly indicating that the effects of UFA on LH secretion are not mediated by direct activation of GPR. Because the nuclear factors PPAR are major targets of FA and PPARγ ligands were recently shown to regulate gonadotropin gene promoter activity in LβT2 cells (37, 38), we also evaluated PPARγ potential contribution. Treatment of LβT2 cells with the selective PPARγ antagonist GW9662 (10 μM) did not interfere with linoleate-induced LH release (Fig. 5A), indicating that these receptors do not mediate the observed effects of UFA.

We next examined the contribution of lipid β-oxidation in linoleate effect using Etomoxir (100 μM), an in-

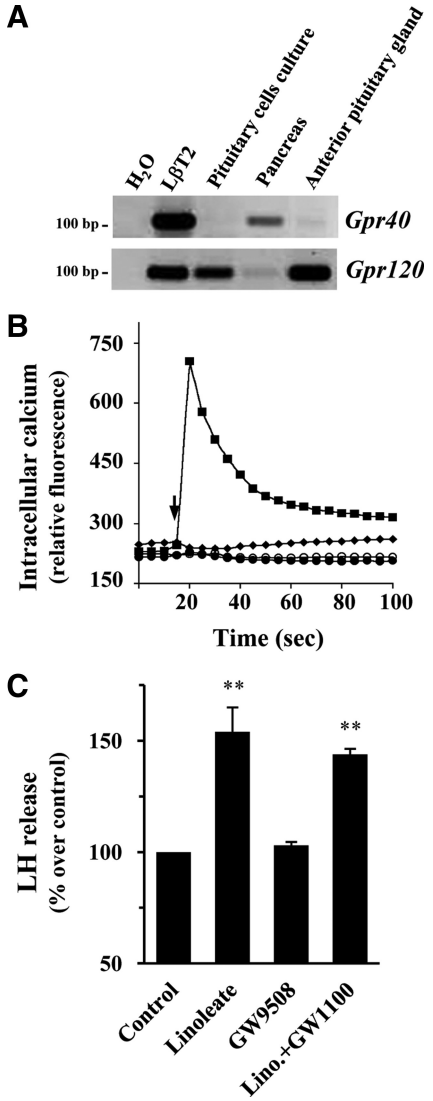

FIG. 4. Linoleate does not signal through membrane receptors GPR40 or GPR120 to stimulate LH release in gonadotrope cells. A, Characterization of GPR40 and GPR120 in anterior pituitary gland and gonadotrope cells. The expression of membrane receptors, GPR40 and GPR120, was analyzed by RT-PCR in LβT2 or cultured anterior pituitary cells as well as in rat anterior pituitary gland, as indicated in *Materials and Methods*. Rat pancreas was used as a positive control tissue for *GPR40* transcripts. B, Linoleate does not mobilize intracellular Ca^{2+} in LβT2 cells. LβT2 cells were loaded for 1 h with a Ca^{2+} fluorescent dye and then treated with 200 μM linoleate (○), 1 mM methyl-β-cyclodextrin (●) or vehicle (◆). Fluorescent signals were recorded every 1.5 sec for 4 min and plotted every 5 sec during the first 100 sec. The *arrow* indicates the onset of the stimulation. GnRHa (10 nM) was used as a positive control (■). The figure shows a representative experiment of four independent experiments. C, Membrane FA receptors do not mediate the stimulatory effect of linoleate on LH secretion. LβT2 cells were treated for 4 h with 200 μM linoleate alone, 10 μM of the GPR agonist, GW9508, or with linoleate in combination with 10 μM of the GPR40/120 antagonist, GW1100. LH release during the following 4 h was then evaluated. The results are expressed as percentage over basal and are the mean ± SEM of four experiments. **, $P \leq 0.01$ compared with control cells. Lino., Linoleate.

hibitor of the outer mitochondrial membrane enzyme carnitine palmitoyl transferase I controlling FA entry into mitochondria. Treatment of LβT2 cells with Etomoxir did

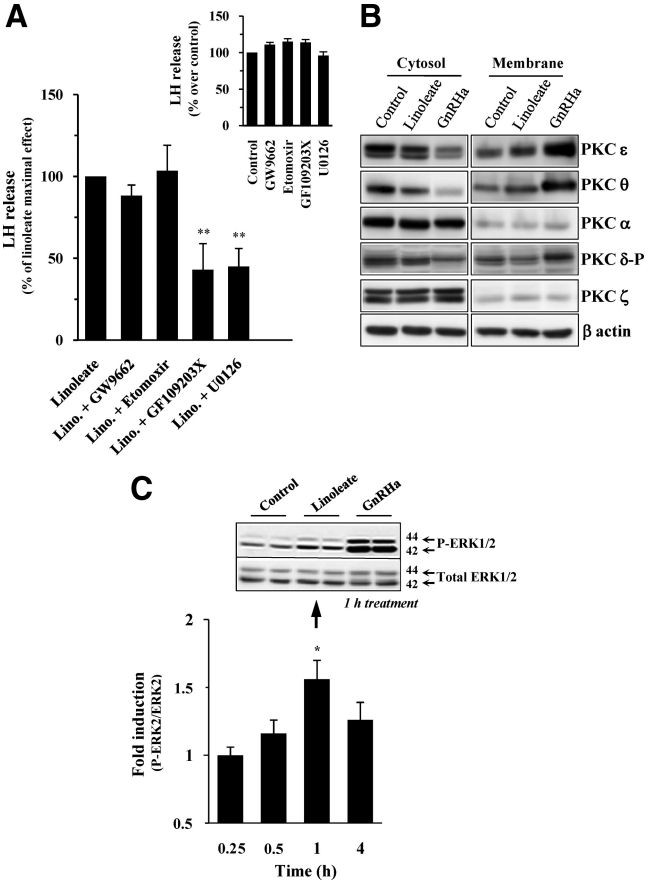

FIG. 5. Evidence for PKC and ERK pathway involvement in linoleate-induced LH release in LβT2 cells. A, PKC and ERK pathways mediate the stimulatory effect of linoleate on LH secretion. LβT2 cells were incubated for 4 h with 200 μM linoleate alone or in combination with 20 μM GW9662 (PPAR antagonist), 100 μM Etomoxir (FA oxidation inhibitor), 10 μM U0126 (MEK1/2 inhibitor), or 4 μM GF109203X (PKC inhibitor). LH release was measured by ELISA after an additional 4-h incubation period. Results are normalized to the effects of drugs alone, expressed as percentage of linoleate maximal effect, and are the mean ± SEM of four to six experiments. **, $P \leq 0.01$ compared with linoleate-treated cells. *Inset*, Effects of the drugs on basal LH release. None of the drugs significantly affected LH release (111 ± 3; 115 ± 4; 114 ± 4; 96 ± 5% over basal for GW9662, Etomoxir, GF109203X, and U0126, respectively). B, Linoleate activates novel PKCε and -θ. LβT2 cells were stimulated by 200 μM linoleate for 30 min. PKCα, -ε, -θ, and -ζ subcellular distribution and PKCδ phosphorylation state were studied by immunoblotting as described in *Materials and Methods*. Antibodies dilution was 1:1000; 1:10,000; 1:2000; 1:10,000; 1:5000; 1:500 for P-ERK1/2, PKCα, θ, ε, ζ and phospho-δ (Thr505), respectively. The figure shows representative blots of three independent experiments. GnRHa (100 nM) stimulation was used as positive control. β-Actin was used as a loading control, and a representative blot is shown. C, Time course of ERK1/2 activation by linoleate in LβT2 cells. LβT2 cells were incubated for increasing periods of time (0.25 to 4 h) with 200 μM linoleate. Phosphorylated forms of ERK1/2 and total ERK were analyzed by immunoblotting as described in *Materials and Methods*. Treatment of cells with GnRHa (10 nM) was used as positive control for ERK activation. *Inset*, illustration of a representative blot, performed in duplicate, obtained at 1 h. Data are expressed as fold induction over respective basal level and are the mean ± SEM of five experiments. *, $P \leq 0.05$ compared with unstimulated control cells at each time. Lino., Linoleate.

not prevent linoleate action, indicating that its β-oxidation is not a necessary step (Fig. 5A). None of the inhibitors tested significantly affected LH release (Fig. 5A, *inset*). In contrast, treatment of cells with the PKC inhibitor GF109203X (4 μM) significantly reduced the stimulatory effect of linoleate on LH release (43 ± 16% of linoleate maximal effect). LβT2 cells express PKC isoforms belonging to the three known PKC families, conventional (α, β2,) novel (δ, ε, θ), and atypical (ζ) (6, 39). Therefore, we next identified linoleate-activated PKC isoforms by evaluating their translocation to the plasma membrane after a 30-min linoleate treatment. Stimulation with GnRHa, known to induce a selective translocation of several isoforms, was used as a control. The distribution of conventional PKCα or atypical PKCζ was affected neither by GnRHa treatment, as previously described in gonadotrope cells (39), nor by linoleate (Fig. 5B). In contrast, linoleate treatment induced a clear redistribution of novel PKCε and PKCθ from the cytosolic to the membrane fractions (Fig. 5B), although to a lower extent compared with GnRHa. Because PKCδ is reported to be mainly regulated by phosphorylation in gonadotrope cells (40), we evaluated linoleate-induced phosphorylation of PKCδ using a phospho-PKCδ (Thr505) antibody. No phosphorylation could be observed after linoleate treatment (Fig. 5B) whereas GnRHa treatment induced an upper shift reflecting higher phosphorylation state of PKCδ. Treatment with palmitate affected neither subcellular redistribution nor phosphorylation of the PKC isoforms studied (data not shown). Finally, we analyzed the involvement of the ERK cascade using the MEK1/2 inhibitor U0126 (10 μM). U0126 treatment also significantly blocked the action of linoleate (45 ± 11% inhibition) (Fig. 5A). Consistent with these findings, we found that UFA promote activation of the ERK pathway in LβT2 cells. Indeed, both linoleate and oleate increased phosphorylation of ERK1/2 as compared with control cells. Maximal activation (1.6 fold) of ERK2 in response to linoleate was observed after 1 h treatment (Fig. 5C), and a similar pattern was observed for ERK1 (data not shown). ERK1/2 were similarly activated by oleate (~1.5 fold at 1 h; data not shown). In contrast, the PI3K/Akt pathway appears not recruited by UFA in LβT2 cells because the PI3K inhibitor, LY 294002 (25 μM), did not interfere with linoleate-induced LH release and no phosphorylation of Akt could be detected after linoleate treatment (data not shown).

UFA counteract GnRH-induced LH release and Ca^{2+} mobilization in gonadotrope cells

A possible effect of FA on GnRH-induced LH secretion by gonadotrope cells was first evaluated in primary cultures of rat pituitary cells. As illustrated in Fig. 6A, treat-

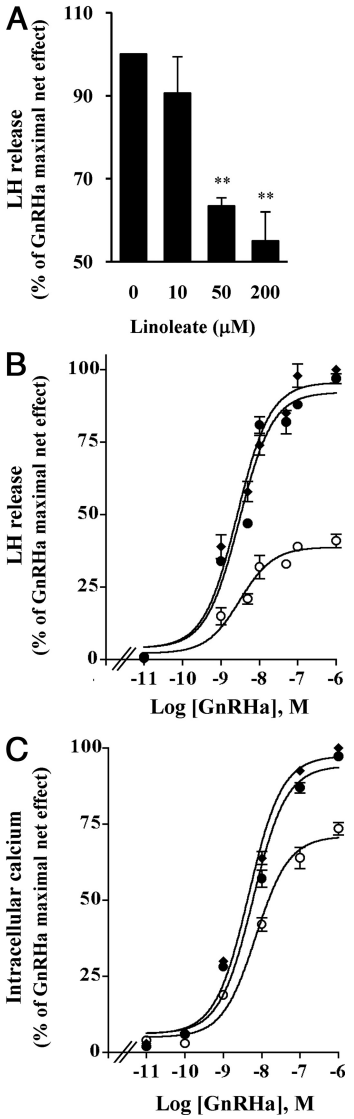

FIG. 6. Linoleate counteracts GnRH-induced Ca^{2+} mobilization and LH release in gonadotrope cells. A, Primary cultures of rat anterior pituitary cells were incubated or not with increasing concentrations (10–200 μM) of linoleate for 4 h and extensively washed. The effect of GnRHa (0.1 nM) on LH release was then measured after a 4-h stimulation period. Results are expressed as the percentage of GnRHa maximal net effect and are the mean ± SEM of three experiments. **, $P \leq 0.01$ compared with control cells. B, LβT2 cells were incubated or not (♦) with 200 μM linoleate (○) or 1 mM methyl-β-cyclodextrin (●) for 4 h, extensively washed, and then stimulated by increasing concentrations of GnRHa (10^{-11} to 10^{-6} M) during 4 h. GnRHa-induced LH release is expressed as percentage of GnRHa maximal net effect in control cells and is the mean ± SEM of three experiments. C, Effect of linoleate on GnRH-induced Ca^{2+} mobilization in LβT2 cells. LβT2 cells were incubated or not (♦) with 200 μM linoleate (○) or 1 mM methyl-β-cyclodextrin (●) for 4 h, washed, and loaded for 1 h with a Ca^{2+} fluorescent dye. Cells were then stimulated with increasing concentrations of GnRHa (10^{-11} to 10^{-6} M), and fluorescence emissions were measured during 4 min. Increases in intracellular Ca^{2+} were observed as sharp peaks above basal fluorescent level typically 3 sec after the addition of GnRHa. Increases of intracellular Ca^{2+} were expressed as percentage of GnRHa maximal net effect in control cells. Data are mean ± SEM of three independent experiments performed in triplicate.

ment of cells with increasing concentrations of linoleate significantly inhibited, in a dose-dependent manner, GnRHa-induced LH secretion. Maximal inhibition (63 ± 4%) was observed with 50 μM linoleate and was not further significantly increased with higher concentrations. A similar inhibitory pattern was observed with oleate (maximal inhibition of 56 ± 5%; data not shown). In contrast, treatment with palmitate or methyl-β-cyclodextrin did not affect cell responsiveness to GnRHa (data not shown). These inhibitory effects were also analyzed in LβT2 cells by measuring the effects of 200 μM linoleate on LH response to increasing concentrations of GnRHa (Fig. 6B). GnRHa induced a dose-dependent increase in LH release that was significantly reduced by linoleate (~60% inhibition) whereas methyl-β-cyclodextrin had no effect. In contrast, linoleate treatment did not affect EC_{50} values (3.1 *vs.* 2.7 nM with and without linoleate, respectively). Moreover, we evaluated linoleate effect on GnRH-induced Ca^{2+} mobilization (Fig. 6C). GnRHa increased intracellular Ca^{2+} in a concentration-dependent manner in LβT2 cells as expected, with a maximal activation at 10^{-6} M GnRHa (3-fold increase over control). As observed for GnRH-induced LH secretion, treatment with linoleate but not methyl-β-cyclodextrin significantly decreased GnRHa-induced Ca^{2+} mobilization (~30% inhibition) without affecting the EC_{50} value (5.9 *vs.* 4.6 nM with and without linoleate, respectively).

Discussion

The physiology of reproduction is closely intertwined with energy balance, but the precise mechanisms underlying the dialogue between these two functions are far from being fully understood. There is a growing amount of data indicating that FA-enriched diets interfere with reproductive processes (26, 41). However, the precise sites of action of FA and notably a possible direct effect of FA on pituitary has only been poorly investigated. In the present work, we demonstrate, for the first time to our knowledge, that FA can directly regulate rodent pituitary gonadotrope function both *in vivo* and *in vitro*.

We have used a rat model for targeting a FA increase into the brain without any associated increase in systemic plasma, thus limiting potential indirect effects of FA notably by perturbation of gonadal function. Triglycerides were administered through the arterial carotid toward the brain and, in particular, to the hypothalamus-pituitary complex, together with heparin to stimulate hydrolysis of triglycerides into FA. *In vivo* central infusion of FA upregulated pituitary *Lhb* and *Cga* transcript levels (Fig. 1). No significant increase in hypothalamic GnRH and Kiss-1 transcript levels could be detected. The level of GnRH transcripts was however elevated after lipid infusion although this did not reach significance. Because FA have been reported to regulate hypothalamic neurons (23), their potential effect on GnRH or afferent neurons should deserve further attention. Our present data suggest, however, that GnRH secretion may not be greatly enhanced by a 24 h-FA central infusion. Indeed, levels of pituitary transcripts of GnRH receptor and NOS I enzyme, known to be up-regulated by GnRH *in vivo* (5, 42), were unaffected by triglyceride infusion. In contrast, a direct stimulatory effect of FA on gonadotrope cells was evidenced in the *in vitro* studies. Indeed, we show here that short-term treatment with the monounsaturated oleate or the polyunsaturated linoleate significantly enhances LH release in both rat pituitary and mouse LβT2 cells (Fig. 2). We provide evidence that this can be explained, at least partly, by an enhancement of LH synthesis. Indeed, in both cell types, UFA increased *Lhb* transcript levels as revealed by real-time RT-PCR studies (Fig. 3). This study provides the first evidence that the gene coding for LHβ subunit is an additional gene in the growing list of genes regulated by FA. Because it has been well established that the α-glycoprotein subunit is expressed in excess and weakly regulated in the pituitary gonadotropes (43), regulation of LHβ subunit directly impacts the amount of LH dimer. Thus, FA-induced *Lhb* increase probably explains, at least in part, the observed enhancement of LH secretion. The observed differences between *in vivo* and *in vitro* regarding *Cga* transcripts and LH secretion may rely on different concentrations and/or nature of FA delivered to gonadotrope cells and also by the complexity of *in vivo* regulation of pituitary function that probably interfere with FA action.

When reaching the pituitary gland, FA encounter a diverse and heterogeneous population of endocrine cells. The action of FA on GH secretion by somatotrope cells has been the subject of extensive investigations. *In vivo* elevation of circulating FA levels was reported to decrease GH secretion in several species including humans (44, 45). A direct inhibitory effect on GH release was demonstrated in porcine pituitary cells (46) whereas some stimulatory effects of UFA on LH secretion were also reported. All these data strongly suggest that FA may regulate LH and GH secretion by the pituitary in an opposite manner. Oleate was previously reported to be ineffective in inducing prolactin release by rat primary cell cultures (44). Interestingly, we show here in the same model that FA do not interfere with ACTH, as reported elsewhere (47) or TSH release. Altogether, this demonstrates that the effect of UFA on pituitary hormone secretion is restricted to a subpopulation of cells, gonadotrope and somatotrope. Contrasting with oleate or linoleate, we report here that palmi-

tate is ineffective in either inducing LH release or increasing gonadotropin subunit transcript levels. This suggests that saturated and unsaturated acids have differential effects on gonadotrope activity, as already reported for other biological processes. For example, the potency of UFA to promote glucose-induced insulin release increases with the degree of insaturation (48). Furthermore, oleate and linoleate were shown to promote cell proliferation whereas palmitate induced apoptosis in breast cancer cells (49). The precise explanation for this differential potency remains to be clarified.

How FA and/or their derivatives can interfere with cell functioning has been the subject of an extensive attention during recent years, and diverse mechanisms have been characterized in organs such as the pancreas, muscle, adipose tissue, or brain. Because no information was available so far in the anterior pituitary, we have evaluated the potential contribution of several of these described mechanisms in gonadotrope cells. Despite the fact that β-oxidation mediates some of the FA effects, including their central regulation of glucose-induced insulin secretion (50), we show here, based on pharmacological inhibition of CPT1 with Etomoxir, that FA β-oxidation is not involved in the stimulatory effect of FA on LH secretion (Fig. 5). We then characterized for the first time two membrane FA receptors, GPR120 in rat pituitary and murine LβT2 gonadotrope cells, and GPR40 in LβT2 cells. However, despite being expressed in LβT2 cells, our results based on pharmacological manipulation of these receptors with specific antagonist (GW1100) or agonist (GW9508) ruled out their involvement in the stimulatory effect of linoleate on LH secretion (Fig. 4). Given that both receptors increase Ca^{2+} levels in different cells (51), the fact that UFA did not elicit any rapid mobilization of intracellular Ca^{2+} in LβT2 cells further substantiates their noninvolvement. The ligands activating GPR40 and 120 in gonadotrope cells as well as the precise roles of these receptors remain thus to be determined. Our results also ruled out the contribution of PPARγ in the observed effects of UFA.

Several studies have reported that FA or their derivatives can activate different kinases and hence interfere with cellular signaling pathways. Among these kinases, PKC have been identified for years as a target of FA (52). Because in gonadotrope cells, PKC is a key kinase involved in basal as well as GnRH-induced LH release and biosynthesis (3), we therefore hypothesized that activation of selective PKC isoforms could be essential in UFA regulation of LH secretion. In support of such a hypothesis, we demonstrate here, based on translocation and phosphorylation studies, that linoleate activates novel PKCε and -θ isoforms without affecting PKCδ, conventional PKCα, or atypical PKCζ in LβT2 cells. Furthermore, involvement of PKC was evidenced by the fact that the broad-spectrum PKC inhibitor *GF109203X* significantly blocks linoleate stimulation of LH secretion. Activation of PKC has already been reported to mediate the action of FA on the secretion of several hormones, including insulin, glucagon, or glucagon-like peptide-1. Furthermore, FA activation of novel PKC isoforms ε and/or θ contributes to FA-induced insulin resistance in several peripheral tissues (30). More recently, alteration of PKCθ subcellular localization by palmitate was reported to mediate hypothalamic insulin resistance (53). Altogether, this suggests that FA-induced activation of novel PKC isoforms is an important molecular event underlying the action of FA in different cell types. Because ERK pathway is mainly recruited via PKC in gonadotrope LβT2 cells, we also evaluated the contribution of this pathway. We reported, based on phosphorylation and pharmacological inhibition studies, that ERK pathway is indeed involved in the observed UFA effects (Fig. 5).

Interestingly, we also report here that oleate and linoleate inhibit GnRHa-stimulated LH release in both rat primary cell cultures and LβT2 cells (Fig. 6). Once again, this effect appears specific to UFA. This result is rather intriguing given the observed stimulatory effect of UFA on basal LH release in these cells. We provide evidence in this study that linoleate decreases GnRH signaling as evidenced by a significant reduction in GnRHa-induced calcium mobilization in LβT2 cells. This was also reported for oleate-inhibitory effect on pituitary TRH receptor signaling (54). Multiple mechanisms may mediate UFA inhibition of GnRH action in gonadotrope cells. GnRH-induced increase in intracellular Ca^{2+} is partly due to stimulation of voltage-gated calcium channels that can be inhibited by UFA (55). In addition, modifications of membrane biophysical properties by UFA may impair the signaling of GnRH-R, preferentially localized in specialized plasma membrane microdomains (56). Furthermore, we provide evidence here that UFA activate two different kinases in gonadotrope cells, PKC and ERK1/2, that may phosphorylate several elements of GnRH-R signaling leading to desensitization. Alternatively, because PKC and ERK mediate the stimulatory effect of GnRH on LH secretion, their mobilization by UFA may hinder GnRH signaling in gonadotrope cells. The challenge is now to analyze the precise targets of UFA in GnRH-signaling pathway.

Several lines of evidence have established that pituitary gonadotrope cells are direct targets of metabolic signals such as insulin or the adipokines, leptin and adiponectin. Our work, by demonstrating that circulating FA can interfere with basal and GnRH-dependent gonadotrope activity in rodents, highlights the existence of lipid sensing in

the pituitary. Altogether, our observations underline the relevant role of pituitary in the control exerted by nutritional signals and in particular by nutrients on reproductive function.

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