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Production of leukotrienes in gonadotropin-releasing hormone-stimulated pituitary cells: Potential role in luteinizing hormone release

(arachidonate 5-lipoxygenase/gonadoliberin/lutropin)

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ABSTRACT **Gonadotropin-releasing hormone (GnRH) stimulated the formation of two major metabolites of the 5-lipoxygenase pathway, leukotriene (LT) B_4 and LTC_4, as well as luteinizing hormone (LH) release in primary cultures of rat anterior pituitary cells. Several lines of evidence suggested the presence of a GnRH-dependent pituitary endocrine system in which LTs act as second messengers for LH release: (*i*) GnRH-dependent LT formation was observed within 1 min and immediately preceded GnRH-induced LH release, whereas exogenous LTs stimulated LH release at low concentrations; (*ii*) the dose responses of GnRH-induced LT production and LH release were similar and both effects required the presence of extracellular Ca^{2+} ions; (*iii*) GnRH-induced LH release was blocked by up to 45% following the administration of several LT receptor antagonists; (*iv*) LTE_4 action on LH secretion was entirely abolished by LT receptor antagonists; and (*v*) an activator of protein kinase C acted synergistically with LTE_4 to induce LH release. The major source of LT formation in the pituitary cell cultures appeared to be the gonadotrophs, as shown by GnRH receptor desensitization experiments. The results demonstrate the presence of a GnRH-activatable 5-lipoxygenase pathway in anterior pituitary cells and provide strong support for the hypothesis that LTs play a role in LH release in the GnRH signaling pathway.**

Gonadotropin-releasing hormone (GnRH), a decapeptide that is formed in the hypothalamus, stimulates the synthesis and secretion of gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone, in a small subset of pituitary cells (1, 2). Besides its established physiological role in the control of gonadotropin release, GnRH and its analogues administered exogenously are used to treat patients afflicted with infertility and estrogen-dependent diseases. In recent years attempts have been made to elucidate the mechanisms of action of GnRH, and it has been shown that GnRH activates a phosphatidylinositol 4,5-bisphosphate-specific phospholipase C (3). This reaction generates diacylglycerol, the endogenous activator of protein kinase C (4, 5), and inositol 1,4,5-trisphosphate, which induces a rise in the concentration of intracellular free Ca^{2+} ions (6). The GnRH-dependent activation of LH secretion is accompanied by arachidonic acid (AA) release from cellular phospholipids (7). Moreover, exogenous leukotrienes (LTs) at picomolar concentrations have been shown to induce LH release in primary cultures of rat anterior pituitary cells (8, 9).

The second-messenger role of AA release in the mechanism of action of GnRH remains to be defined. Rat anterior pituitary cells mainly metabolize free AA by oxidative pathways (10), and GnRH-stimulated release of LH can be attenuated by inhibition of 5-lipoxygenase (11). Therefore, it has been proposed that LTs and possibly other oxygenated products of AA metabolism are physiological mediators of LH release and other brain functions (8, 12, 13). Although no evidence has been presented that pituitary cells express the 5-lipoxygenase pathway, Lindgren *et al.* (14) reported that several brain tissues, including the hypothalamus, can form LTs. However, the source of LTs in the anterior pituitary, their functional role, and whether LTs can act as second messengers in the signaling pathway of GnRH remain unanswered questions. In the work detailed below we used monolayer cultures and a superfusion system of primary anterior pituitary cells in an attempt to elucidate the role of LTs in pituitary cell function. Our results demonstrate that pituitary cells form LTB_4 and LTC_4 in response to GnRH and provide strong support for the hypothesis that LTs act as second messengers in the GnRH signaling pathway.

MATERIALS AND METHODS

Cell Culture. Single-cell suspensions were prepared from anterior pituitary glands of adult female Sprague–Dawley rats (200 g; Savo-Ivanowas, Kisslegg, F.R.G.) as described (15). Cells were maintained on Cytodex beads (Pharmacia) at a cell/bead ratio of 20:1 in Earle's medium 199 (Biochrom, Berlin) supplemented with 60 μg of gentamicin per ml and 10% horse serum. Superfusion experiments were performed using perifusion columns (Pharmacia) with 2×10^7 cells per column. The superfusion medium was supplemented with 20 mM Hepes and the flow rate was 0.5 ml/min. Down-regulation of GnRH receptors was accomplished by incubation of pituitary cells in the presence of the GnRH agonist buserelin [pyroGlu-His-Trp-Ser-Tyr-D-Ser(*t*-Bu)-Leu-Arg-Pro-NHEt] at 0.1 μM for 24 hr as described (16). Viability tests have shown that buserelin does not have toxic effects on pituitary cells under conditions used in these experiments.

The LT antagonists FPL 55,712, ICI 198,615, and LY 171,883 were kindly provided by Fisons (Loughborough, U.K.), ICI, and Eli Lilly, respectively. FPL 55,712 and LY 171,883 were dissolved in double-distilled water, and ICI 198,615 was dissolved in dimethyl sulfoxide (Merck) at a final concentration of 0.01%. GnRH (Peninsula Laboratories) was dissolved in medium 199. In superfusion experiments, fractions were collected every 2 min and assayed for LH content with the radioimmunoassay (RIA) kit (anti-rLH-S-10; rLH-

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Abbreviations: AA, arachidonic acid; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; LT, leukotriene; PMA, phorbol 12-myristate 13-acetate; RP, reversed-phase.

I-8; rLH-RP-2) provided by the National Hormone and Pituitary Program (Baltimore). Data are presented as LH concentration (ng/ml per 2×10^7 cells). Statistical significance was determined by using Student's t test to compare peak hormone values. Data are expressed as means of quadruplicate dishes ± SD.

Incubation Conditions. Measurement of LT synthesis in rat anterior pituitary cells was performed precisely as described by Borgeat and Samuelsson (17) for polymorphonuclear leukocytes. Briefly, rat anterior pituitary cells were taken up in Ringer's solution (pH 7.0, 37°C) at a density of 10^6 cells per ml. Cells were incubated for 15 min in the presence of 50 μM AA that had been dissolved in 10 μl of ethanol or in the presence of ethanol as control. Then L-cysteine that had been dissolved in 10 μl of phosphate-buffered saline was added to a final concentration of 10 mM, and the incubation was continued for 2 min. Finally, Ca^{2+} ionophore A23187 (20 μM) dissolved in 10 μl of ethanol or ethanol alone (control) was added, and the incubation was continued for an additional 10 min. At this point the reaction was stopped by addition of ice-cold ethanol to give an ethanol concentration of 70% (vol/vol).

Reversed-Phase High-Pressure Liquid Chromatography (RP-HPLC). LTs were chromatographed on octadecyl silica gel chromatography columns (J. T. Baker) that had been conditioned with methanol according to Powell (18). The samples were dried under negative pressure and chromatographed on μBondapak RP columns (3.9 × 300 mm; 5-μm particles; Waters) with methanol/water (65:35, vol/vol) acidified to pH 5.7 with acetic acid as described by Mathews et al. (19) and Habenicht et al. (20). The RP-HPLC system was equipped with an M-510 pump and a single-wavelength UV detector operating at 280 nm (Waters). LTs were eluted isocratically at a flow rate of 1 ml/min. The retention times of LTC_4 and LTB_4 were 23 and 35 min, respectively. Concentrations of LTC_4 and LTB_4 were determined by RIA (21). Fractions containing LTC_4 and LTB_4, respectively, were collected, dried under nitrogen, and taken up in RIA buffer (10 mM KH_2PO_4/10 mM Na_2HPO_4/150 mM NaCl, pH 7.4). Water samples subjected to exactly the same analytical procedure as the biological samples ranged between 8 and 15 pg/ml. Procedural losses were monitored by adding tracer LTs. Total recoveries for LTB_4 ranged between 39% and 54% and for LTC_4 between 35% and 56%. Data are expressed as means of quadruplicate dishes ± SD.

RESULTS

LTB_4 and LTC_4 Formation in Anterior Pituitary Cells. Previous studies have shown that several brain tissues form LTs in response to Ca^{2+} ionophore and AA (14) and that cultured pituitary cells respond to picomolar amounts of exogenous LTs by releasing LH (8, 9, 22). However, there was no evidence that pituitary cells possess a functional 5-lipoxygenase pathway, and the cellular origin and potential physiological role of LTs in the LH release pathway remained to be determined.

Table 1. Formation of LTB_4 and LTC_4 and LH release in anterior pituitary cells

Addition(s)	LTB_4, pg	LTC_4, pg	LH, ng
None	12.1 ± 1.5	1.8 ± 0.3	2.3 ± 0.3
AA	16.5 ± 3.2	1.9 ± 0.1	24 ± 1.8
AA + A23187	728 ± 127	178 ± 23	182 ± 64

Monolayer cells (2×10^7 per dish) were incubated in medium 199 without addition or with AA (50 μM) or Ca^{2+} ionophore A23187 (20 μM) plus AA for 10 min. Formation of LTs was detected by RIA after HPLC. LH was determined by RIA. Data represent means of quadruplicate dishes ± SD and are normalized per 10^6 cells.

Table 2. Down-regulation of GnRH receptors in anterior pituitary cells leads to desensitization of GnRH-dependent and Ca^{2+} ionophore-dependent LT production and LH release

Condition	LTB_4, pg	LTC_4, pg	LH, ng
	GnRH-dependent		
Control	738 ± 41	119 ± 38	167 ± 45
Desensitized	16 ± 1	4 ± 0	27 ± 3
	Ca^{2+} ionophore-dependent		
Control	728 ± 127	178 ± 23	182 ± 64
Desensitized	21 ± 6	4 ± 0	48 ± 8

Cultured anterior pituitary cells were preincubated without (control) or with (desensitized) 0.1 μM buserelin. LT formation and LH release in response to 0.1 μM GnRH or 20 μM Ca^{2+} ionophore in the presence of 50 μM AA was then determined. Data represent means of quadruplicate dishes ± SD and are normalized per 10^6 cells.

To test whether pituitary cells express the 5-lipoxygenase pathway, we incubated primary cultures of anterior pituitary cells in the presence or absence of maximal concentrations of the Ca^{2+} ionophore A23187 and unesterified AA. In the absence of Ca^{2+} ionophore but in the presence of AA the cells produced little or no LT. However, when the same cultures were incubated in the presence of both Ca^{2+} ionophore and AA, synergistic LT synthesis was observed (Table 1). These results demonstrated that cultured pituitary cells possess a functional 5-lipoxygenase pathway. Moreover, pituitary cells that had been stimulated to produce LTs in response to Ca^{2+} ionophore and AA showed a strong concomitant release of LH (Table 1). These results are of interest in relation to published evidence that low concentrations of added LTs or 5-lipoxygenase stimulate LH release (8, 9, 23) and show that receptor-independent LT production, induced by Ca^{2+} ionophore and AA, is also associated with LH release.

Since only ≈10% of the total anterior pituitary cell population express GnRH receptors, we next determined the cellular source of pituitary LTs. Two types of experiments were performed to achieve this aim. In the first type, we incubated the total population of anterior pituitary cells with GnRH or Ca^{2+} ionophore in the presence of AA and deter-

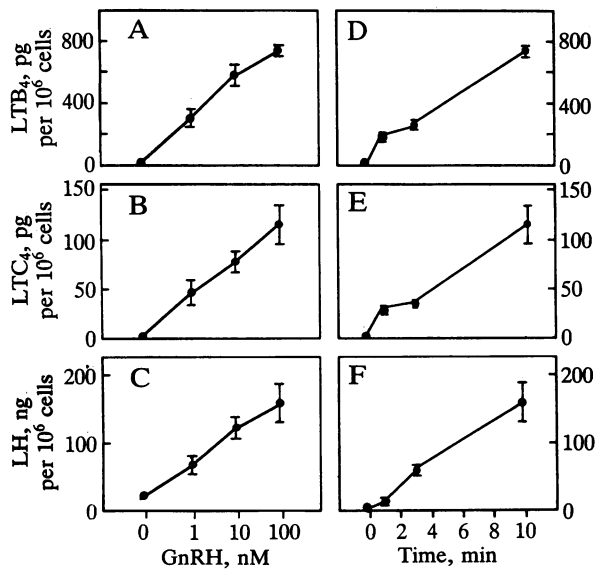

FIG. 1. Concentration and time dependence of GnRH-induced LTC_4 and LTB_4 formation and LH release in cultured anterior pituitary cells. The cells were cultured and incubated with AA as described under *Materials and Methods*. At time zero, 0, 1, 10, or 100 nM GnRH was added and the reaction was stopped 10 min later (*A–C*) or at time zero, 100 nM GnRH was added and the reaction was stopped as indicated (*D–F*). The concentrations of LTs and LH were determined. Data represent means of quadruplicate dishes ± SD.

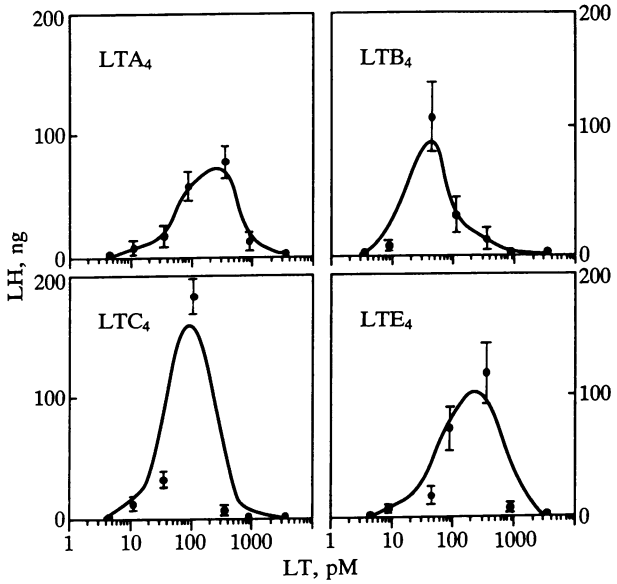

FIG. 2. LT-dependent LH release in superfused rat anterior pituitary cells. Perifusion columns containing 2 × 10^7 anterior pituitary cells were prepared as described under *Materials and Methods*. LTs were added for 4 min to the superfusion medium at 60-min intervals and the concentration of LH in perifusate was determined. The effect of LT on LH secretion was calculated as area under the curve and expressed as ng of LH. Data represent means of quadruplicate experiments ± SD.

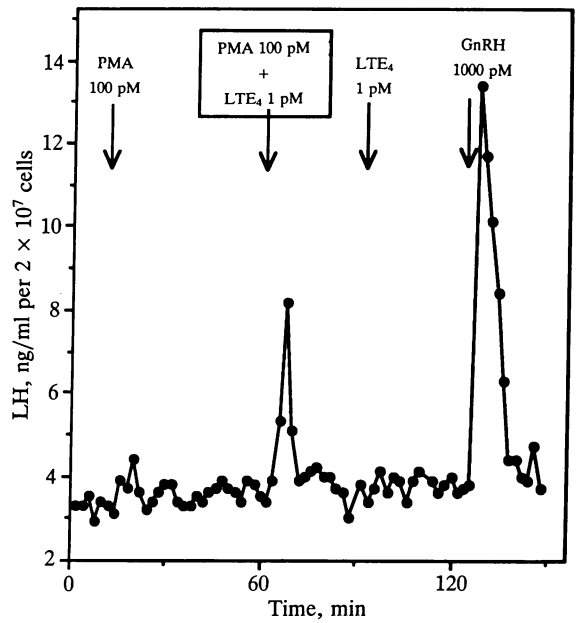

FIG. 3. Phorbol ester- and LTE_4-dependent LH release in superfused anterior pituitary cells. Perifusion columns were prepared and PMA and LTE_4 were added as described under *Materials and Methods*.

mined subsequent LT formation as well as LH release. We found that GnRH strongly stimulated LT production at similar concentrations as were required for LH release (Table 2 and see below). Furthermore, the magnitude of GnRH-dependent LT synthesis was almost identical to that of Ca^{2+} ionophore-dependent LT synthesis, which we consider to represent the total 5-lipoxygenase pathway activity of all pituitary cells.

In the second type of experiment, GnRH receptor desensitization was used to determine the relation between expression of GnRH receptors and LT synthesis in the total anterior pituitary cell population. The GnRH agonist buserelin was used to specifically desensitize GnRH receptors (16). Desensitized cells were unable to respond to the addition of GnRH by producing significant amounts of LTs and releasing LH. Moreover, the desensitized cells were also unable to respond to Ca^{2+} ionophore (Table 2).

Kinetics of GnRH-Dependent LT Synthesis and Concentration Dependence of LT-Induced LH Release in Anterior Pituitary Cells. To study the relation between the effects of GnRH on LT formation and LH release in more detail, we determined the kinetics and concentration dependence of both effects. We found that GnRH-induced LH release and LTC_4 and LTB_4 synthesis paralleled each other in a time- and concentration-dependent manner. LTC_4 dramatically induced LH release, amounting to a 7-fold increase within 10 min (Fig. 1). The same type of experiments have been carried out using the GnRH superagonist buserelin (results not shown). Buserelin mimicks the effect of GnRH on LT formation in anterior pituitary cells and is more potent in its action on LT synthesis than GnRH. Furthermore, LTB_4 and to a lesser extent LTA_4 and LTE_4 were also strong inducers of LH release (Fig. 2). These results confirmed and extended published data by Hulting *et al.* (8) and Lindgren *et al.* (22) demonstrating that LTC_4 can induce LH release in pituitary cell cultures. Our finding that LTs other than LTC_4 are also

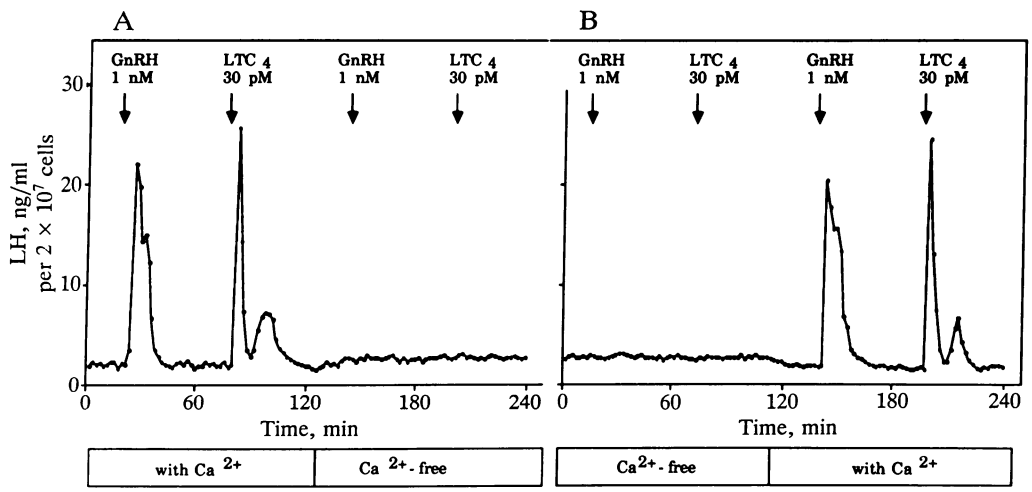

FIG. 4. GnRH- and LTC_4-dependent LH release requires the presence of extracellular Ca^{2+} ions. Perifusion columns containing 2 × 10^7 anterior pituitary cells were prepared and 1 nM GnRH or 30 pM LTC_4 was added at the indicated times. The superfusion medium containing 1.5 mM Ca^{2+} was changed to Ca^{2+}-free medium at 130 min (*A*) or from Ca^{2+}-free to Ca^{2+}-containing medium at 110 min (*B*).

potent inducers of LH release in the pituitary might be explained by the use of the superfusion system employed in these types of experiments (see Fig. 2). In contrast to the maintenance of cells in culture dishes, this system allows continuous monitoring of LH release in response to repetitive additions of different agonists or culture media under standardized conditions and thus provides the opportunity to detect relatively small changes in the concentration of LH in the perifusion medium.

The Effects of GnRH and Exogenous LTC_4 on LH Release Require Extracellular Ca^{2+}, and LT-Dependent LH Release Is Synergistically Enhanced by Phorbol Ester. To further study the relation between GnRH-dependent and LT-dependent LH release, we examined the effects of phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, on LH release in the presence or absence of LTE_4. PMA at 0.1 nM or LTE_4 at 1 pM had no effect on LH release. However, when PMA and LTE_4 were added together, significant LH release was observed (Fig. 3). This raises the possibility that GnRH and LTE_4 share a common metabolic pathway in the LH release reaction. Further evidence for this possibility was provided by the striking Ca^{2+} dependence of both GnRH- and LTC_4-dependent LH release (Fig. 4).

Several LT Receptor Antagonists Inhibit the Effects of GnRH and LTE_4 on LH Release. To study whether LTs can act as second messengers for the GnRH signaling pathway, anterior pituitary cells were preincubated in the presence of LT receptor antagonists before addition of GnRH. All three LT receptor antagonists significantly inhibited the effect of GnRH on LH release in superfused rat anterior pituitary cells (Fig. 5). The inhibitory effect on the secretion of LH was 38.2%, 45.4%, and 21.9% for FPL 55,712, ICI 198,615, and LY 171,883, respec-

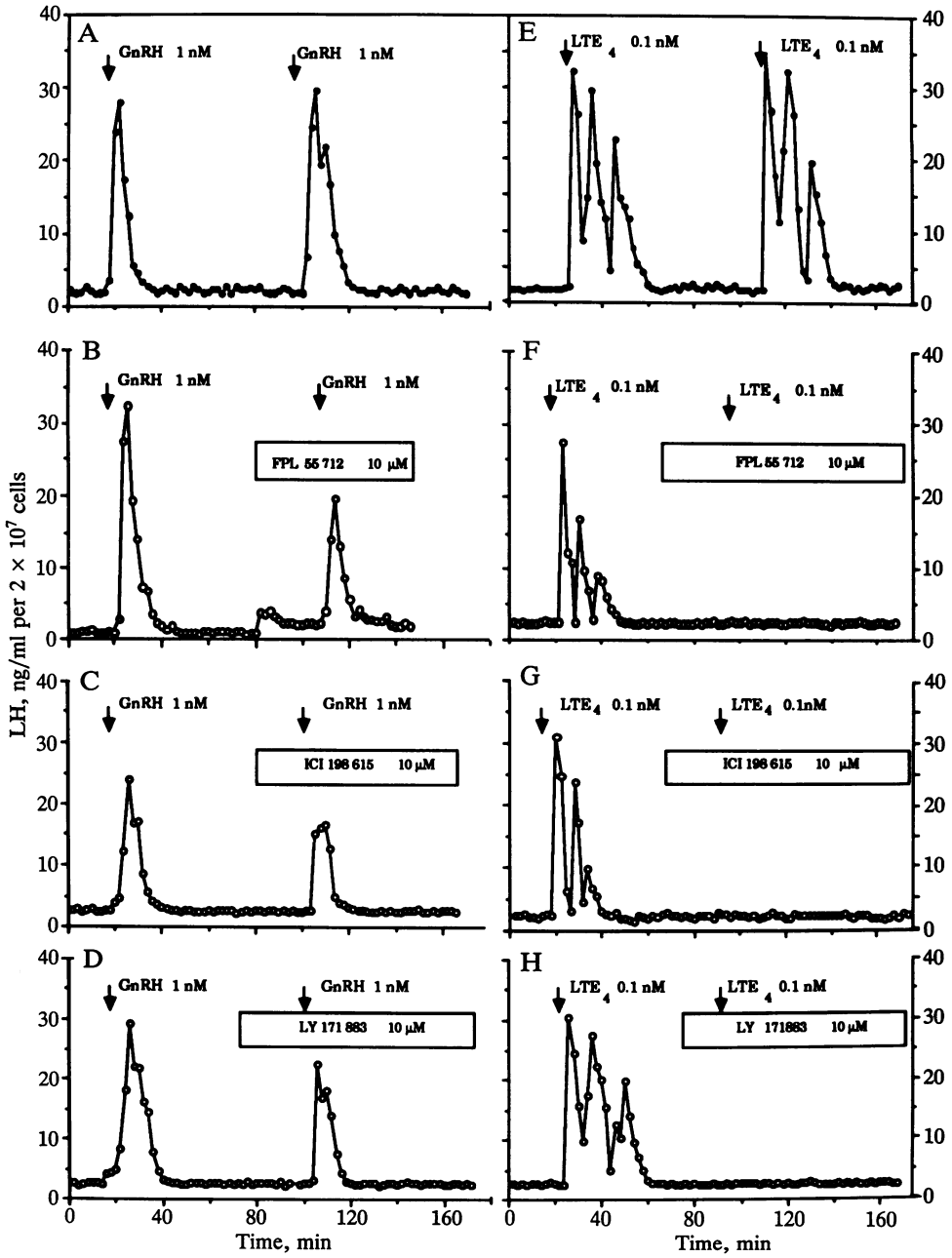

FIG. 5. Inhibition of GnRH- and LTE_4-dependent LH release by LT receptor antagonists. Perifusion columns were prepared and 1 nM GnRH or 0.1 nM LTE_4 was added. LT receptor antagonist [FPL 55,712 (*B* and *F*), ICI 198,615 (*C* and *G*), or LY 171,883 (*D* and *H*)] at 10 μM was added to the superfusion medium 30 min before addition of GnRH (*B–D*) or LTE_4 (*F–H*). This is a representative experiment of three performed.

tively. The administration of LTE_4 (0.1 nM) resulted in a striking triphasic release of LH. All three LT receptor antagonists abolished LTE_4-induced LH release (Fig. 5).

DISCUSSION

Our results can be summarized as follows: rat anterior pituitary cells possess a functional 5-lipoxygenase pathway, and gonadotrophs are the major source of LT formation in this tissue. GnRH and its analogue buserelin stimulate LT formation and LH release, and both effects depend on extracellular Ca^{2+} ions and require similar concentrations of agonist (24). In addition, GnRH-dependent LT release precedes LH release, and LT receptor antagonists significantly block the effect of GnRH on LH release. Exogenously administered LTs, at picomolar concentrations, induce LH release, and the effect of submaximal concentrations of LTs on LH release is synergistically enhanced by an activator of protein kinase C. When considered together these results provide strong support for the hypothesis, originally proposed by Hulting *et al.* (8, 12), that LTs are involved in the control of LH release in the pituitary. In particular, we have identified GnRH receptor-expressing cells as the major source of LT synthesis in the anterior pituitary and provided an experimental framework for the further study of the role of LTs in endocrine tissues.

The conclusion that cells that express GnRH receptors are a major source of LT synthesis in the pituitary is based on two types of experiments. First, the magnitude of GnRH-dependent LT formation is almost identical with receptor-independent (i.e., Ca^{2+} ionophore-dependent) LT formation (Table 1). Second, that GnRH receptor-desensitized cells showed a >95% inhibition of LT synthesis in response to Ca^{2+} ionophore indicates that expression of the 5-lipoxygenase pathway in the pituitary is largely confined to a small subpopulation of pituitary cells, namely, those that express GnRH receptors. Further studies are required to elucidate the mechanism and functional significance of GnRH-dependent down-regulation of the 5-lipoxygenase pathway.

While it is very difficult to prove that LTs function as second messengers in the GnRH signaling pathway, several lines of evidence strongly support such a role: (*i*) the potency of LTC_4 induction of LH release is several orders of magnitude higher than that of its physiological agonist, GnRH (Fig. 4); (*ii*) the kinetics of GnRH-dependent LT synthesis are consistent with a second-messenger role in the LH release pathway; and (*iii*) most important, several LT receptor antagonists are capable of significantly blocking the effect of GnRH on LH release.

LTs are believed to act in an autocrine and/or paracrine fashion *in vivo* (25), and our LT receptor antagonist data are compatible with this hypothesis. AA and LTs may act together with other second-messenger pathways, such as phospholipase C and protein kinase C (3, 4, 5, 26), since LT receptor antagonists only partially block the effect of GnRH on LH release and since activators of protein kinase C act synergistically with LTE_4 to induce LH release (Fig. 3). The signaling pathways induced by GnRH may, however, also synergize with those induced by LTE_4, since data from others demonstrated an activation of protein kinase C by LTs (27).

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