Gonadotropin Releasing Hormone Activates the Lipoxygenase Pathway in Cultured Pituitary Cells: Role in Gonadotropin Secretion and Evidence for a Novel Autocrine/Paracrine Loop†

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ABSTRACT: The formation and role of arachidonic acid (AA) and its metabolites during gonadotropin releasing hormone- (GnRH-) induced gonadotropin secretion were investigated in primary cultures of rat pituitary cells. Prelabeled cells ([^{3}H]AA) responded to GnRH challenge with increased formation (about 2-fold) of the leukotrienes LTC_4, LTD_4, and LTE_4 as well as 5- and 15-eicosatetraenoic acids (5- and 15-HETE) as identified by HPLC. Formation of leukotrienes and 15-HETE was further verified by specific radioimmunoassays. No significant increase in the formation of 12-HETE or of the cyclooxygenase products prostaglandin E (PGE) and thromboxane A_2 by GnRH was noticed. Addition of physiological concentrations of LTC_4 enhanced basal LH release, while subphysiological concentrations of LTC_4 (10^{-15}–10^{-12} M) inhibited GnRH-induced LH release by about 35% ($p < 0.02$). Using specific lipoxygenase inhibitors L-656,224 and MK 886, we found inhibition of GnRH-induced LH release by about 40% at concentrations known to specifically inhibit the 5-lipoxygenase pathway. The peptidoleukotriene receptor antagonist ICI 198,615 inhibited LTC_4- and LTE_4-induced LH release and surprisingly also the effect of GnRH on LH release by 40%. The data strongly suggest a role for AA and its lipoxygenase metabolites in the on/off reactions of GnRH upon LH release. The data also present a novel amplification cycle in which newly formed leukotrienes become first messengers and establish an autocrine/paracrine loop.

The hypothalamic neurohormone, gonadotropin releasing hormone (GnRH),[1] regulates the synthesis and release of the gonadotropic hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH). GnRH, a Ca^{2+}-mobilizing hormone, activates phosphoinositide turnover (Naor et al., 1986; Morgan et al., 1987) to provide inositol 1,4,5-trisphosphate (IP_3) that release Ca^{2+} from intracellular stores (Berridge & Irvine, 1989; Guillemete et al., 1987; Naor et al., 1988a) and diacylglycerol (DG), the physiological activator of protein kinase C (PKC) (Nishizuka, 1988; Naor et al., 1985a; Hirota et al., 1985). In addition to IP_3 and DG, GnRH stimulation of anterior pituitary cells in culture results also in the release of arachidonic acid (AA) to the medium (Naor & Catt, 1981; Naor et al., 1983, 1985b). Lipoxygenase metabolites of AA were implicated as potential second messengers in cell signaling (Naor & Catt, 1981; Naor et al., 1983, 1985b; Naor, 1991; Needleman et al., 1986; Piomelli et al., 1987). Indeed it was previously shown that AA and several of its lipoxygenases or epoxygenase products stimulate LH release in pituitary cells in culture and in superfusion (Naor & Catt, 1981; Naor et al., 1983, 1985b; Naor, 1991; Snyder et al., 1983; Hulting et al., 1985; Kiesel et al., 1987a,b, 1991; Chang et al., 1987). The reported order of potency of the lipoxygenase metabolites in stimulating LH release in vitro was the following: $LTC_4 > LTA_4 > LTE_4 >>$ 15-HETE > 5-HETE > AA (Kiesel et al., 1987a,b, 1991). In addition, phospholipase A_2 and first generation of lipoxygenase inhibitors (NDGA, ETYA, and BW 755C) reduced LH responses to a GnRH challenge (Naor et al., 1983, 1985b). Thus, although indirect evidence suggested a possible role for AA and its metabolites in GnRH action, no direct demonstration of formation of active metabolites of AA by the neurohormone under physiological conditions has been demonstrated. We therefore examine here the formation by GnRH of oxygenated arachidonate metabolites and their relevance to the exocytotic response of GnRH. We also examine here the potential role of AA metabolites as second messengers in cell signaling.

Experimental Procedures

Materials

GnRH was purchased from Peninsula Laboratories (San Carlos, CA). Trypsin, soybean trypsin inhibitor, DNase, horse serum, and arachidonic acid were from Sigma (St. Louis, MO). Medium 199 was from Biological Industries (Beit

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[1] Abbreviations: GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; LTB_4, LTC_4, LTD_4, LTE_4, leukotriene B_4, C_4, D_4, E_4 respectively; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; AA, arachidonic acid; PGE, prostaglandin E; PKC, protein kinase C; DG, diacylglycerol; MK 886, 3-[1-(*p*-chlorobenzyl)-5-isopropyl-3-(*tert*-butylthio)indol-2-yl]-2,2-dimethylpropanoic acid; L-656,224, 7-chloro-2-[(4-methoxyphenyl)methyl]-3-methyl-5-propyl-4-benzofuranol; ICI 198,615, [1-[[2-methoxy-4-[[(phenylsulfonyl)amino]carbonyl]phenyl]-methyl]-1*H*-indazol-6-yl]carbamic acid cyclopentyl ester; ETYA, 5,8,11,14-eicosatetraynoic acid; NDGA, nordihydroguaiaretic acid; BW 755 C, 3-amino-1-[3-(trifluoromethyl)phenyl-2-pyrazoline hydrochloride; IP_3, inositol 1,4,5-trisphosphate; RP-HPLC, reverse-phase high-pressure liquid chromatography; RIA, radioimmunoassay.

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Ha'Emek, Israel). Radioimmunoassay (RIA) reagents for LH were kindly provided through the NIDDK, NIH, and the National Hormone and Pituitary Program (NHPP) of the University of Maryland School of Medicine (Baltimore, MD). [^{3}H]Arachidonic acid was from New England Nuclear (240 Ci/nmol, 8880 GBq/mmol; Boston, MD). GnRH antagonist, D-pcl-Phe^2-D-Trp^{3,6}-GnRH was kindly provided by Dr. D. Coy (Tulane University, New Orleans, LA). Hydroxy fatty acids (15-, 12-, and 5-HETE), peptidoleukotrienes (LTC_4, LTD_4, LTE_4), and the lipoxygenase inhibitors L-656,224, 7-chloro-2-[(4-methoxyphenyl)methyl]-3-methyl-5-propyl-4-benzofuranol, and MK 886, 3-[1-(*p*-chlorobenzyl)-5-isopropyl-3-(*tert*-butylthio)indol-2-yl]-2,2-dimethylpropanoic acid, were the kind gift of Dr. Ford-Hutchinson of Merck Frosst Canada Inc. (Point Duval, Quebec, Canada). The peptidoleukotriene antagonist ICI 198,615, [1-[[2-methoxy-4-[[(phenylsulfonyl)amino]carbonyl]phenyl]methyl]-1*H*-indazol-6-yl]-carbamic acid cyclopentyl ester, was kindly provided by Dr. D. Aharoni of ICI Americas, Wilmington, DE. Antibodies to prostaglandin E (PGE) and thromboxane B_2 were kindly provided by Dr. F. Kohen of the Weizmann Institute of Science, Rehovot, Israel.

Methods

Preparation of Pituitary Cell Cultures. Labeling experiments: Pituitary glands from 21-day-old Wistar-derived rats were used for cell preparation as previously described (Naor et al., 1986, 1988a). The cells were kept in suspension (1×10^6 cells/mL) for 3–4 days. On the day of the experiment, the cells were washed (2×10^7 cells/tube) and incubated in medium 199 with [^{3}H]arachidonic acid (4 μCi/mL) at 37 °C for 10 min. Following another wash, the cells were incubated for the specific length of time with various concentrations of GnRH in 1 mL of medium 199 + 0.1%. The reaction was stopped by the addition of 10 volumes of the ice-cold extraction mixture chloroform/methanol (2:1). The samples were then acidified with 0.05 N H_2SO_4 and left at 4 °C for 30 min. Following vigorous mixing, the organic phase was separated by a 5-min spin at 1000 rpm. The organic phase was collected and the solvent was evaporated under a N_2 stream. The samples were dissolved in the HPLC solvent solution prior to analysis.

HPLC: Arachidonic acid metabolites were separated by reverse-phase HPLC (LiChrospher column 100, 5 μm, RP-18, Merck) with a solvent system of methanol/water/acetic acid (71:29:0.02 v/v), pH 5.6, as described by McColl et al., (1986). Metabolites were eluted at a flow rate of 1.5 mL/min. By this method prostaglandins and leukotrienes elute at the first 10 min, while the hydroxy acids elute between 11 and 22 min. The eluent was collected by a fraction collector every 30 s, and radioactivity was monitored by a scintillation counter and compared to authentic standards (internal and external) and detected by an on-line absorbance detector at 280 nm for peptidoleukotrienes and 234 nm for the hydroxy acids.

Experiments for RIA Analysis. In another set of experiments, cells [(3–4) $\times 10^7$/tube] were incubated with or without GnRH (1 nM) for 10 min. Samples were extracted as described above. AA metabolites were then separated by HPLC. The eluent was collected (0.75 mL/tube), dried under N_2, redissolved in PBS containing 3% horse serum, and sonicated for 10 min in ice. Peptidoleukotrienes were determined by specific RIA as described by Zor et al. (1987). RIA for 15-HETE was performed on dried samples redissolved in assay buffer as described by Natarajan et al. (1988). RIAs for prostaglandin E and thromboxane B_2 (a metabolite of thromboxane A_2) were performed as previously described (Naor et al., 1975; Ojeda et al., 1979).

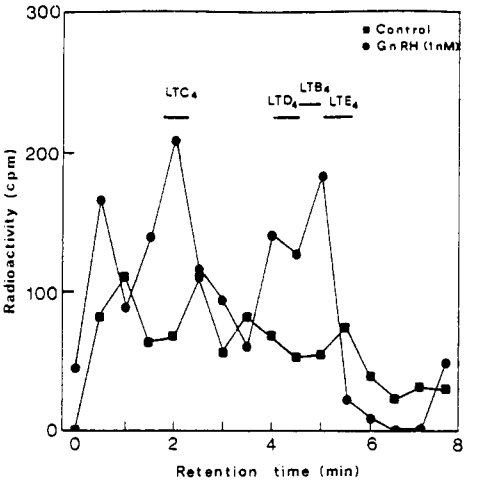

FIGURE 1: RP-HPLC elution profile of GnRH-induced leukotrienes formation. Pituitary cells in suspension (2×10^7) were prelabeled with [^{3}H]AA for 10 min and then washed and incubated with or without GnRH (1 nM, 10 min). Following extraction, samples were dried under N_2 and redissolved in mobile phase, and leukotrienes were separated by RP-HPLC (elution buffer: methanol/H_2O/HAc, 71:29:0.02 v/v; flow rate of 1.5 mL/min). The eluent was collected; radioactivity was counted and compared to authentic standards of leukotrienes (internal and external), detected by an on-line UV detector (at 280 nm).

LH release: Cells (3×10^5 per well) were plated in 24-well plates. After 3–4 days in culture, cells were washed twice and then incubated for 30 min in medium 199 containing 0.02% BSA with various concentrations of leukotrienes or GnRH. In another set of experiments, cells were incubated for 2 h with GnRH in the presence of various concentrations of LTC_4. In yet another set of experiments, the washed cells were preincubated with various concentrations of the 5-lipoxygenase inhibitors L-656,224, MK 886, or the leukotriene receptor antagonist ICI 198,615. GnRH (1 nM) was added after 30 min for an additional period as indicated. The medium was collected and stored frozen for the LH RIA. Results are expressed in terms of RP-3 preparation of the NIDDK of NIH.

RESULTS

Upon exposure of [^{3}H]AA-prelabeled pituitary cells in primary culture to GnRH, several oxygenated metabolites of the lipoxygenase pathway of AA were formed (Figures 1 and 2). Separation by reverse-phase HPLC revealed that arachidonate metabolites with retention times that correspond to those of authentic standards of LTC_4, LTD_4, LTE_4, and perhaps LTB_4 were elevated in response to the GnRH challenge (Figure 1). LTB_4 separated poorly from LTD_4 under the conditions described here, and its formation therefore needs further verification. In addition, metabolites with a retention time that corresponds to those of 15-HETE and 5-HETE were also observed to be elevated by GnRH (Figure 2). Another metabolite of the lipoxygenase pathway, namely 12-HETE, showed no consistent elevation by GnRH as measured by HPLC combined with RIA.

Formation of the peptidoleukotrienes by GnRH was further confirmed by analysis of the HPLC corresponding peaks by specific RIAs (Figure 3). GnRH stimulated LTD_4 and LTE_4 formation by 190% and 235%, respectively. Since LTC_4 is rapidly converted to LTD_4 and LTE_4, elevation of LTD_4 and LTE_4 indicates a parallel increase in LTC_4. As with the peptidoleukotrienes, formation of 15-HETE was also confirmed by specific RIA and was found to increase from below de-

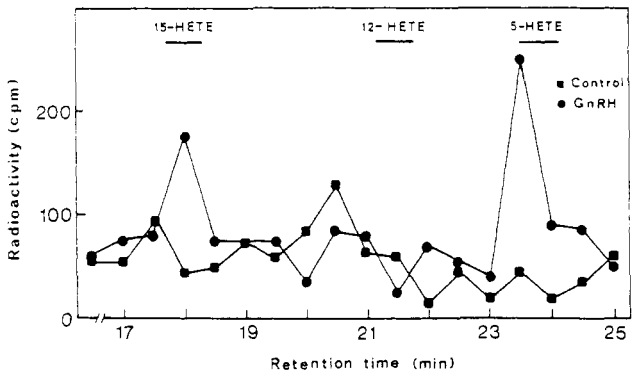

FIGURE 2: RP-HPLC chromatogram of GnRH-induced hydroxy acids formation. Pituitary cells in suspension [(1.5–2.0) × 10^7] were prelabeled with [^{3}H]AA for 10 min and then washed and stimulated with or without GnRH (1 nM, 10 min). Samples were extracted and separated by RP-HPLC as described in the legend to Figure 1. The radioactivity was counted and the elution time was compared to those of authentic standards detected by an on-line UV detector (235 nm).

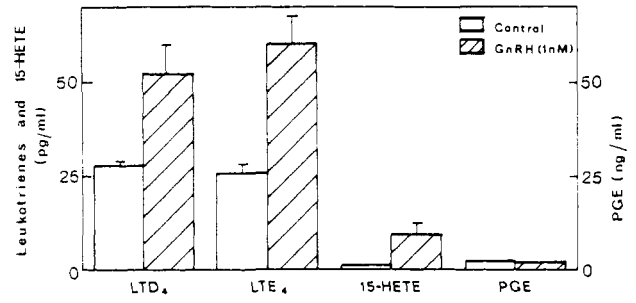

FIGURE 3: GnRH-induced eicosanoid formation, assayed by specific RIA. For 15-HETE and peptidoleukotriene assays, pituitary cells in suspension [(4–5) × 10^7] were incubated with or without GnRH (1 nM, 10 min). Lipoxygenase metabolites were extracted as above and dried. Following separation by RP-HPLC (as described in the legend to Figures 1 and 2), the collected eluent was dried and redissolved in assay buffer and then assayed by specific RIA for 15-HETE and peptidoleukotrienes. For the PGE assay, 2 × 10^7 cells were stimulated with or without GnRH (1 mM, 10 min). Medium was collected and assayed for PGE by specific RIA. Results are the mean of triplicates from a representative experiment.

tection levels by RIA to 29 ± 7 pM in response to GnRH (1 nM) challenge. Hence, the fold of stimulation of 15-HETE levels is larger than that for LTD_4 and LTE_4. The cyclooxygenase product prostaglandin E was not affected by GnRH (1 nM) (7.2 ± 0.5 and 6.5 ± 0.5 nM for control and GnRH-stimulated cells, respectively). Another metabolite of the cyclooxygenase pathway thromboxane B_2 (an indicator of thromboxane A_2) was undetected in the pituitary cells in the presence or absence of GnRH.

The GnRH effect on arachidonate metabolites formation was time-dependent as shown here for LTC_4 and 15-HETE formation (Figure 4). LTC_4 was elevated in response to GnRH (1 nM) as early as 1 min after stimulation and reached its peak at around 4 min of incubation. Unlike LTC_4, the increase in 15-HETE formation in response to GnRH challenge was detected mainly around 10 min of incubation. Yet another metabolite, 5-HETE, reached its peak of formation after 20 min of exposure to GnRH (1 nM) (not shown). The GnRH effect was dose-dependent as exemplified here for 15-HETE formation (Figure 5). Both LTC_4 and 15-HETE showed the same dose dependency with maximal response at about 1 nM GnRH.

Pretreatment of the cells with the potent GnRH antagonist D-pcl-Phe^2-D-Trp^{3,6}-GnRH blocked GnRH formation of identified AA metabolites as 15- and 5-HETE, as well as LTC_4 and LTE_4 (not shown).

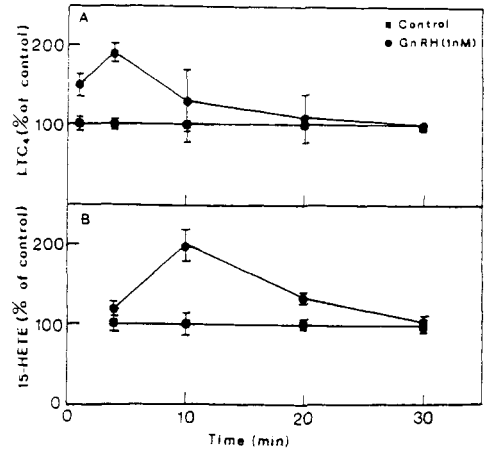

FIGURE 4: Time response curve of GnRH-induced LTC_4 and 15-HETE formation. Pituitary cells in suspension were preincubated with [^{3}H]AA, washed, and challenged with GnRH (1 nM) for the indicated times. The extracted samples were dried and separated by RP-HPLC as described in the legends to Figures 1 and 2. (A) Time response of LTC_4 formation. (B) Time response of 15-HETE formation. Data (mean ± SEM, n = 5) are expressed as a percentage of the control. Basal disintegration per minute levels at 30 min for LTC_4 and 15-HETE were 362 and 1824, respectively.

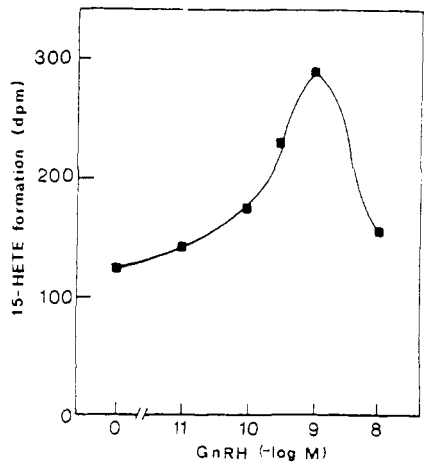

FIGURE 5: Dose–response curve of 15-HETE formation. Prelabeled pituitary cells were stimulated with increasing concentrations of GnRH for 10 min. Samples were extracted and dried under N_2. Following RP-HPLC separation using an authentic 15-HETE standard, radioactivity was counted. The curve is composed of three separate experiments (SEM < 15%).

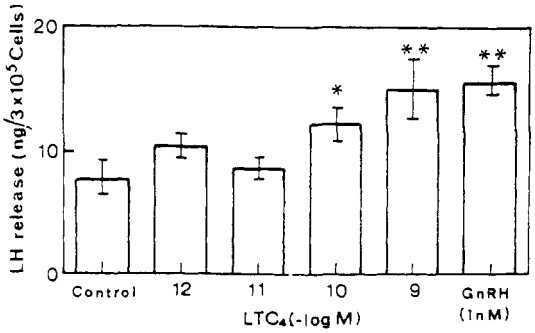

FIGURE 6: Effect of LTC_4 on LH release. Cultured pituitary cells (3 × 10^5/well) were incubated in medium 199 + 0.2% BSA and increasing concentrations of LTC_4. At the end of 30 min of incubation, the medium was collected and assayed for LH by RIA. The points are the means ± SEM of four separate experiments, each in triplicates. Symbols: (*) $p < 0.05$; (**) $p < 0.01$.

We then proceeded to investigate the potential role of AA metabolites as second messengers in GnRH action. Stimulation of pituitary cells with LTC_4 for 30 min enhanced the release of LH in a dose-dependent manner (Figure 6). Similar

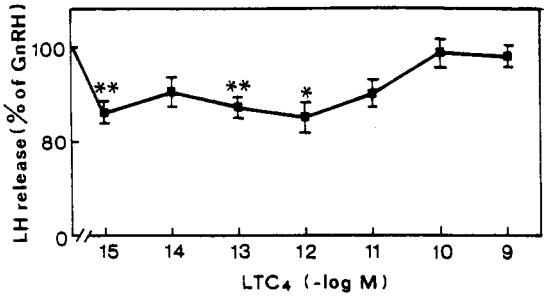

FIGURE 7: Effect of LTC_4 on GnRH-induced LH release. Pituitary cells in culture (3×10^5/well) were incubated with GnRH (1 nM) in the presence of increasing concentrations of LTC_4. At the end of the 2-h incubation period, the medium was collected and assayed for LH by RIA. The points are means ± SEM. Symbols: (*) $p < 0.05$; (**) $p < 0.01$. LH levels were 14.5 ± 2.9 and 54 ± 12 ng/3×10^5 cells for the control and GnRH-stimulated cells, respectively.

Table I: Effect of Lipoxygenase Inhibitors and Leukotriene Antagonist on Early Phase of GnRH-Induced LH Release[a]

		LH release [ng/(3×10^5 cells·30 min)]		
drug	control	GnRH (1 nM)	GnRH (1 nM) + drug	% of GnRH
L-656,224 (2.9 μM)	12.9 ± 0.4	19.1 ± 2.2	16.8 ± 1.2	63
MK 886 (2 μM)	5.0 ± 0.4	22.7 ± 1.2	16.3 ± 0.7	64
ICI 198,615 (1.8 μM)	9.4 ± 1.8	23.0 ± 1.8	16.9 ± 0.8	55

[a] Pituitary cells in primary cultures (3×10^5/well) were preincubated with drugs for 30 min. Following a change of medium, the cells were further incubated with GnRH (1 nM) in the presence or absence of the drug for another 30 min. Medium was collected and assayed for LH. Results are the mean ± SEM of triplicate determinations. The drugs had no effect on basal LH release.

results were noted with LTE_4, while LTD_4 was found ineffective (data not shown). The effect of LTC_4 (100 pM)[2] amounted to about 50% of the GnRH response at 30 min. Paradoxically, subphysiological concentrations of LTC_4 (10^{-15}–10^{-12} M) had no effect on basal LH release, but they partially inhibited (35%, $p < 0.02$) GnRH-induced LH release (Figure 7).

To further confirm that the 5-lipoxygenase pathway is involved in GnRH-induced LH release, we examined the effect of the selective 5-lipoxygenase inhibitors L-656,224 and MK 886 (Belanger et al., 1987; Dixon et al., 1990; Rouzer et al., 1990), on the early (Table I) and late (Figure 8) phases of GnRH-induced LH release. The drugs had no significant effect on basal LH release at 30 min or 2 h of incubation. On the other hand, the drugs affected gonadotroph responsiveness to the GnRH challenge. At a very low dose of inhibitors (1–5 nM), the drugs slightly enhanced the GnRH response (Figure 8), possibly by removing the inhibitory effect of subphysiological doses of LTC_4 and LTE_4. Thereafter, GnRH-induced LH release was inhibited by increasing doses of MK 886 by about 30%. L-656,224 (0.5 μM) inhibited GnRH-induced LH release by about the same extent. The inhibitory effect of MK 886 on GnRH-induced LH release could be restored by the addition of LTC_4 (Figure 9).

To investigate whether the newly formed peptidoleukotrienes might operate as first messengers in cell signaling, we investigated the effect of the peptidoleukotriene receptor antagonist (ICI 198,615) (Snyder et al., 1987; Aharony et al., 1988). We first investigated the effect of ICI 198,615 on leukotriene-induced LH release (Figure 10). Stimulation of LH release

[2] The concentration of LTC_4 which is found in GnRH- (1 nM) stimulated pituitary cells.

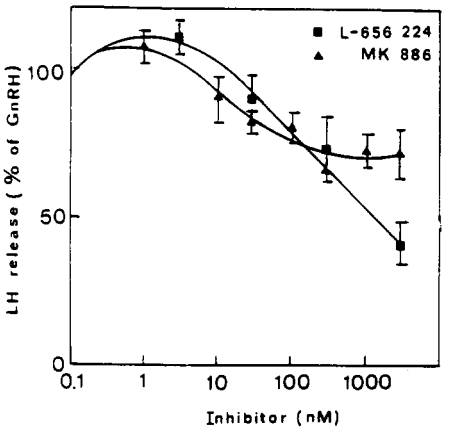

FIGURE 8: Inhibition of GnRH-induced LH release by the 5-lipoxygenase inhibitors L-656,224 and MK 886. Cultured pituitary cells (3×10^5/well) were preincubated with increasing concentrations of the inhibitors for 30 min in medium 199 containing 0.01–0.02% BSA. Cells were then stimulated with GnRH (1 nM) for an additional 2 h. The incubation medium was collected and assayed for LH content. LH values for L-656,224 and MK 886 inhibition curves were 11.6 ● 0.99 and 10.2 ± 1.2 ng/3×10^5 cells for control, respectively, and 30.8 × 2.9 and 32.7 ● 4.1 ng/3×10^5 cells for GnRH-treated cells, respectively. Results are means ± SEM from three experiments, each done in triplicates.

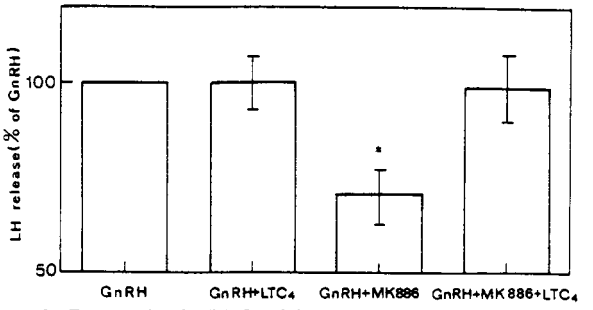

FIGURE 9: Restoration by LTC_4 of GnRH-induced LH release in MK 886 pretreated pituitary cells. Cells in primary culture were preincubated with or without MK 886 (1 μM, 30 min) and then stimulated with GnRH (1 nM, 2 h) in the presence or absence of LTC_4 (100 pM). Medium was collected and stored frozen for LH determination. LH levels were 7.6 ± 0.77 and 28.1 ± 3.3 ng/3×10^5 cells for control and GnRH-stimulated cells, respectively. Results are means ± SEM ($n = 12$). Symbol: (*) $p < 0.05$.

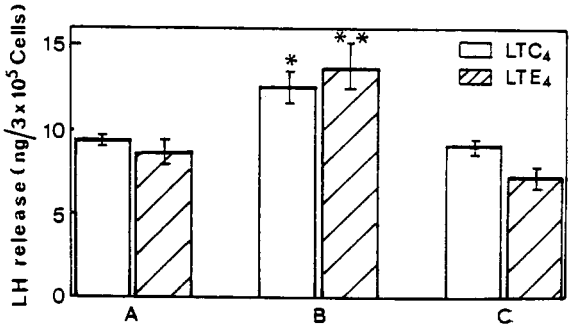

FIGURE 10: Inhibition of LTC_4- and LTE_4-induced LH release by the peptidoleukotriene antagonist ICI 198,615. Cultured pituitary cells were incubated with or without ICI 198,615 for 30 min. Following a change of medium, the cells were stimulated with LTC_4 and LTE_4 in the presence or absence of the antagonist. The incubation medium was collected and assayed for LH by RIA. (A) Control. (B) LTC_4 (100 pM), LTE_4 (1 nM), 30 min. (C) LTC_4 (100 pM) and LTE_4 (1 nM) in the presence of ICI 198,615 (36 and 1.8 μM, respectively). Results are means ± SEM ($n = 6$). Symbols: (*) $p < 0.05$; (**) $p < 0.01$. ICI 198,615 had no effect on basal LH release.

by LTC_4 (100 pM) was abolished by the antagonist (36 μM). Lower concentrations of the antagonist (1.8 μM) abolished LTE_4- (1 nM) induced LH release. We then investigated the

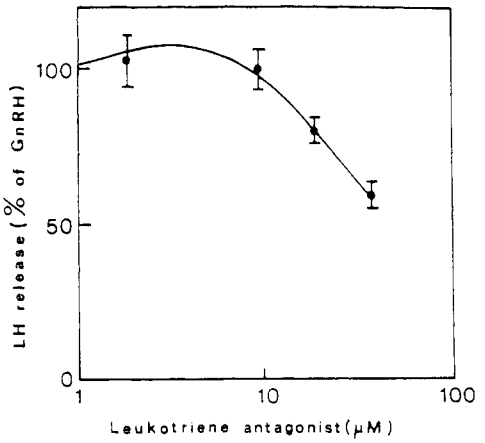

FIGURE 11: Dose–response curve for the inhibition of GnRH-induced LH release by peptidoleukotriene antagonist ICI 198,615. Cultured pituitary cells (3×10^5/well) were preincubated with increasing concentrations of ICI 198,615 for 30 min. Cells were then stimulated with GnRH (1 nM, 2 h). At the end of the incubation period, the medium was collected and assayed for LH by RIA. (LH release values were 14.4 ± 1.6 and 47.2 ± 3.4 ng/3 × 10^5 for the control and GnRH-stimulated cells, respectively). Results are the means ± SEM of at least three experiments done in triplicate wells. ICI 198,615 had no effect on basal LH release.

effect of the antagonist on GnRH-induced LH release (Figure 11). Surprisingly, the antagonist reduced the exocytotic response elicited by GnRH (1 nM) by about 40% at both 30 min and 2 h of incubation (Figure 11 and Table I). Under the conditions described above (Figures 10 and 11 and Table I), ICI 198,615 had no effect on basal LH release at 30 min or after 2 h of incubation.

Discussion

Whereas a large body of literature deals with formation of two second messengers during Ca^{2+}-mobilizing hormones operating via the phosphoinositide turnover [i.e., IP_3 and DG; see Nishizuka (1988) and Berridge and Irvine (1989) for reviews], very little is known about arachidonic acid (AA) as a second messenger in cell signaling [Naor (1991) for a review]. Previous studies have suggested a possible role for AA and/or its lipoxygenase or epoxygenase metabolites in GnRH-induced LH release (Naor & Catt, 1981; Naor et al., 1983, 1985b; Snyder et al., 1983; Hulting et al., 1985; Kiesel et al., 1987a,b, 1991; Chang et al., 1987). On the other hand, the stimulatory effect of the cyclooxygenase product PGE_2 on LH release in vivo was shown to be exerted at the hypothalamic level, with no involvement at the pituitary level in vivo and in vitro (Chobsieng et al., 1975; Ojeda et al., 1979). AA and several of its lipoxygenase metabolites induced LH release in cultured pituitary cells (Naor et al., 1985b; Snyder et al., 1983; Hulting et al., 1985; Kiesel et al., 1987a,b, 1991; Chang et al., 1987), while the first generation of lipoxygenase inhibitors, nordihydroguaiaretic acid (NDGA), 5,8,11,14-eicosatetraynoic acid (ETYA), and 3-amino-1-[3-(trifluoromethyl)phenyl]-2-pyrazoline hydrochloride (BW 755C) inhibited GnRH-induced LH secretion (Naor et al., 1985b; Chang et al., 1987). Nevertheless, the direct demonstration of formation of AA metabolites under physiological conditions by GnRH was lacking. The results of this study demonstrate the formation of biologically relevant metabolites of the lipoxygenase pathway in response to GnRH in cultured pituitary cells. Moreover, the levels of the metabolites achieved by the GnRH challenge (about 100–150 pM for LTC_4 and LTE_4) are sufficient to induce LH release in vitro (see Results). The observation that subphysiological concentrations of LTC_4 (10^{-15}–10^{-12} M) were inhibitory to GnRH action while physiological concentrations ($\sim 10^{-10}$ M) stimulated LH release are surprising but are in line with new evidence on bidirectional control exerted by other messenger molecules involved in GnRH action such as Ca^{2+} and PKC (Leong & Thorner, 1991; Stojilkovic et al., 1991). Both mediators are now thought to participate in the stimulation of LH release as well as the desensitization process (Leong & Thorner, 1991; Stojilkovic et al., 1991). By binding to super-high-affinity receptors, LTC_4 (10^{-15} M) might exert an inhibitory action during GnRH-induced desensitization. On the other hand, binding of LTC_4 (10^{-10} M) to high-affinity receptors evokes stimulation during activation of gonadotrophs by GnRH as indicated here by the lack of additivity with GnRH. The more rapid increase in GnRH-induced LTC_4 as compared to LTD_4 and LTE_4 formation is in line with LTC_4 being the precursor molecule of LTD_4 and LTE_4.

In a distribution study of LTC_4 and monohydroxy fatty acids in rat brain, LTC_4 formation was most prominent in the hypothalamus (Miyamato et al., 1987). Immunohistochemical studies suggested colocalization of GnRH and LTC_4 throughout the median eminence (Hulting et al., 1985). Therefore, a possible neuroendocrine role for peptidoleukotrienes was proposed (Hulting et al., 1985). GnRH stimulation of LTC_4 formation is detected within 1 min of incubation. In view of the fact that GnRH-induced LH release is biphasic (Naor et al., 1982), we propose that LTC_4, the precursor of LTD_4 and LTE_4, is a mediator of the early GnRH effect.

While 5-HETE (Naor et al., 1985b) and 15-HETE (Kiesel et al., 1987a,b) are capable of releasing LH, 12-HETE was found inactive (Naor et al., 1985b; Kiesel et al., 1987a). Yet both 5- and 15-HETE were found to be active at the micromolar range. Since our results demonstrate formation of about 25 pM 15-HETE per 2×10^7 cells by GnRH, and the concentration of 5-HETE following GnRH stimulation as analyzed by HPLC separation is well below the micromolar range needed for LH release, it seems unlikely that 5- and 15-HETE play a direct role in GnRH-induced LH release. A modulatory role for HETEs has been shown recently in rat adrenal glomerulosa cells (Stern et al., 1989). While 12-HETE is involved positively in angiotensin II-induced aldosterone release, 5-HETE in the nanomolar range modulates AII action negatively (Stern et al., 1989).

The fate of 15-HETE generated during GnRH action is not clear. It was shown recently that [^{3}H]-15-HETE was incorporated into the lipid pool of human neutrophils, specifically into phosphatidylinositol (Brezinski & Serhan, 1990). Moreover, as a result of such incorporation, a different profile of metabolites was generated in response to the chemotactic peptide (FMLP) challenge (Brezinski & Serhan, 1990). 15-HETE is an important member of the generated metabolites of AA in GnRH-stimulated gonadotrophs. Hence, incorporation of 15-HETE into phospholipids for reasons not clear at the present time cannot be ruled out. Alternatively, 15-HPETE can be converted to lipoxins (Serhan et al., 1986), and AA and lipoxin A were shown to activate PKCγ and possibly PKCβII as well (Naor et al., 1988b; Shearman et al., 1989; Burns et al., 1990). PKC has been implicated in GnRH action (Turgeon et al., 1984; Naor et al., 1985a; Hirota et al., 1985; Stojilkovic et al., 1988; Davidson et al., 1988; Dan-Cohen & Naor, 1990; Horn et al., 1991), and PKCβII is expressed in the pituitary and is capable of inducing LH release in TPA-downregulated, digitonin-permeabilized cells (Naor, 1990a; Naor et al., 1989). Hence, a complex cross-talk between AA, its lipoxygenase products, and the PKC family

might be operating during Ca^{2+}-mobilizing ligand action in general and GnRH in particular.

The levels of the products of the cyclooxygenase pathway, PGE and thromboxane A_2, were unchanged after GnRH stimulation, although the enzyme is active in pituitary cells (Naor et al., 1975). Thus, the effect of GnRH on peptidoleukotrienes, as well as 5- and 15-HETE formation, is specific and does not reflect an overall increase in AA metabolism as a result of GnRH stimulation of AA release. Hence, GnRH acts at two sites of action: release of AA (Naor & Catt, 1981) and activation of specific lipoxygenase enzymes.

The effect of leukotrienes is relatively rapid as differences are observed between 30 min and 2 h after challenge. Stimulation of LH release in a 30-min protocol by physiologically relevant concentrations of LTC_4 (100–200 pM) amounts to about 50% of the exocytotic response of GnRH. On the other hand, the release during 2 h of incubation is smaller. The results suggest that the majority of contribution of leukotrienes in GnRH action is in the first 30 min of GnRH action. In addition, GnRH mobilizes intracellular stores of Ca^{2+} via IP_3, induces Ca^{2+} influx via voltage-sensitive and -insensitive Ca^{2+} channels, and activates PKC [Naor (1990b) for a review]. Elevated Ca^{2+} levels and PKC might operate in concert with AA metabolites to mediate the first phase of GnRH action. Furthermore, AA metabolites might be the endogenous mediators of Ca^{2+} and/or PKC actions. Further studies are needed to elucidate the complex cross-talk of the various signaling molecules. The later phase of GnRH action, which is detectable after 2 h of incubation, apparently involves influx of Ca^{2+}, lipoxygenase metabolites of arachidonate, and a more prominent role for PKC [Naor (1990b) for a review].

It was previously shown that the general lipoxygenase inhibitors NDGA, ETYA, and BW 755C caused dose-dependent inhibition of GnRH-induced LH release (Naor et al., 1985b). More recently, a 5-lipoxygenase-specific inhibitor became available, namely, MK 886. The new drug binds to five lipoxygenase activating protein (FLAP), an 18-kDa protein that is required for the translocation and activation of the enzyme (Dixon et al., 1990; Rouzer et al., 1990). Thus, by binding to FLAP, MK 886 renders the enzyme inactive. Incubation of pituitary cells with MK 886 inhibited GnRH-induced LH release in a dose-dependent manner with a maximal inhibitory effect of about 30% achieved at 0.3 μM MK 886. The results suggest that 5-lipoxygenase is redistributed to membrane fractions during gonadotroph activation by the neurohormone GnRH. L-656,224 was reported to inhibit 5-lipoxygenase specifically with a reported IC_{50} of 0.4 μM in a cell-free system. At higher concentrations, L-656,224 was reported to inhibit 15- and 12-lipoxygenase (IC_{50} values of 30 μM and 40 μM, respectively) (Belanger et al., 1987). L-656,224 produced a dose-dependent inhibition of GnRH-induced LH release, with about 40% inhibition observed at about 0.5 μM inhibitor. The ability of LTC_4 to fully restore GnRH-induced LH release in the presence of MK 886 and the lack of additivity with GnRH points at the peptidoleukotrienes as the most likely AA metabolite to mediate LH release.

The peptidoleukotriene's receptor antagonist ICI 198,615 was found effective against LTD_4 and LTE_4 in the nanomolar range and against LTC_4 in the micromolar range (Snyder et al., 1987; Aharoni et al., 1988). We noticed consistent inhibition of GnRH-induced LH release by ICI 198,615 in the range of 20-60 μM antagonist in long-term (2 h) challenge, while in short-term (30 min), ICI 198,615 inhibited GnRH-induced LH release effectively with lower concentrations (1.8 μM). It is therefore possible that LTE_4 participates together with LTC_4 in mediating the early phase of LH release, while LTC_4 is involved in the later stage of the release.

The results also suggest that peptidoleukotrienes generated by GnRH exert their effect by the binding of newly formed LTC_4 and LTE_4 to specific receptors on the cell membrane. Once LTC_4 and LTE_4 are formed in the gonadotrophs by GnRH, they are apparently released to bind to the plasma membrane of the same or neighboring cells, where they initiate their own transmembrane signaling. Members of the leukotriene family have been shown recently to induce PI turnover and IP_3 formation (Anderson et al., 1986; Crooke et al., 1989), and we have recently obtained similar results in pituitary cells (manuscript in preparation). Such a mechanism represents an arachidonic acid amplification cycle.

Arachidonate and its lipoxygenase metabolites satisfy the following criteria set by Sutherland for implicating a second messenger role in hormone action (Robison et al., 1971). (I) GnRH stimulates pituitary AA and lipoxygenase metabolite production and gonadotropin release. (II) Stimulation of AA metabolite production by GnRH coincides with gonadotropin secretion. (III) AA and some of its lipoxygenase products mimic the effect of GnRH on LH release. (IV) Inhibitors of GnRH-induced lipoxygenase metabolite production also inhibit GnRH stimulation of LH release. We therefore, propose that AA metabolites of the lipoxygenase pathway are involved as second messengers in GnRH-induced LH release. Since the metabolites mediate only a fraction of the exocytotic response (~40%), the cross-talk of AA metabolites with other identified messengers such as Ca^{2+} and PKC needs to be further investigated.

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