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MECHANISM OF ACTION OF GONADOTROPIN RELEASING HORMONE: ROLE OF LIPOXYGENASE PRODUCTS OF ARACHIDONIC ACID IN LUTEINIZING HORMONE RELEASE

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Summary—The mechanism of action of gonadotropin-releasing hormone (GnRH) upon pituitary luteinizing hormone (LH) secretion has not yet been elucidated, but recent evidence has suggested that arachidonic acid or its metabolites are involved in GnRH action. In cultured rat pituitary cells, arachidonic acid and 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) elicited concentration-dependent release of LH with EC_{50} of about 12 μM. Other lipoxygenase derivatives including 11-, 12- and 15-HETE, had no consistent effect on LH release, and leukotrienes (B_4 and C_4) exerted only minor stimulatory actions on LH release. The lipoxygenase inhibitors nordihydroguaiaretic acid (NDGA), 5,8,11,14-eicosatetraynoic acid (ETYA), and 3-amino-1-(3-trifluoromethyl phenyl)-2-pyrazoline hydrochloride (BW 755C) caused dose-dependent inhibition of GnRH-induced LH release, with IC_{50} values of 5, 8.5, and 175 μM, respectively. In contrast, the cyclooxygenase inhibitor, indomethacin, had a biphasic action on GnRH-stimulated LH release, with potentiation of GnRH action at low doses (up to 25 μM) and no effect at higher concentrations. These findings are consistent with the potential role of a 5-lipoxygenase product of arachidonic acid in the mechanism of action of GnRH on pituitary gonadotropin release.

INTRODUCTION

The control of gonadotropin secretion by the hypothalamic decapeptide, gonadotropin releasing hormone (GnRH[1]), is exerted through the pulsatile action of the peptide upon pituitary gonadotrophs. The initial step of hormone–receptor interaction at the gonadotroph cell membrane is rapidly followed by release of LH and FSH from pituitary storage granules. The releasing hormone binds to specific high-affinity receptor sites located on the plasma membrane of pituitary gonadotrophs [1], and initiates a series of calcium-dependent events [2–5] that culminate in gonadotropin release. Several studies have indicated that neither prostaglandins nor the major cyclic nucleotides (cAMP and cGMP) appear to mediate GnRH-induced gonadotropin release [6–13]. Recently, we have shown that GnRH promotes the turnover of pituitary phosphatidylinositol [14] and stimulates the release of arachidonic acid in cultured pituitary cells [15]. These observations suggested that arachidonic acid or its metabolites could be involved in GnRH action. The rapid turnover of polyphosphoinositides and activation of protein kinase C by diacylglycerol [16] or calcium mobilization by inositol phosphates [17], coupled with arachidonic acid release from phosphoglycerides, are now implicated in signal transduction across the cell membrane. Activation of a calcium-dependent phospholipase A_2, or phosphatidylinositol-specific phospholipase C and diglyceride lipase [18, 19], results in the liberation of free arachidonic acid, which is converted to prostaglandins, prostacyclins, and thromboxanes by the cyclooxygenase pathway, or alternatively is metabolized to HETEs and leukotrienes via the lipoxygenase pathway [20–26]. The production of HETEs and leukotrienes has been shown to be blocked by inhibitors such as NDGA, ETYA and BW 755C. These compounds, and several products of the lipoxygenase pathway, have been employed to evaluate the in-

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[1]Abbreviations used are: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; PG's, prostaglandins; cAMP, adenosine 3',5'-cyclic monophosphoric acid; cGMP, guanosine 3',5'-cyclic monophosphoric acid; 5-HPETE, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 11-HETE, 11-hydroxy-5,8,12,14-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,10,14-eicosatetraenoic acid; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; ETYA, 5,8,11,14-eicosatetraenoic acid; NDGA, nordihydroguaiaretic acid; BW 755C, 3-amino-1-(3-trifluoromethyl phenyl)-2-pyrazoline hydrochloride; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; PI, phosphatidylinositol; PA, phosphatidic acid.

volvement of arachidonic acid metabolites in the mechanism of action of GnRH upon pituitary gonadotropin secretion.

EXPERIMENTAL PROCEDURES

Arachidonic acid metabolites and inhibitors

The hydroxy-fatty acids (5-, 11- and 12-HETE) and leukotriene B_4 were provided by Dr J. Pike of the Upjohn Co., Kalamazoo, MI, and were kept under argon at −70°C. 5-HETE was obtained in the lactone form and was converted to the free acid form by allowing 200 μg of 5-HETE to stand overnight in 1 ml of 2% Na_2CO_3. After reduction of the pH to 5.0, the free acid was extracted into 2 vol of ether and evaporated to dryness, then reconstituted in 0.1% ethanol for use in the cell culture assay. 15-HETE was prepared from arachidonic acid via soybean lipoxygenase and was kindly supplied by Dr R. W. Bryant. Leukotrienes B_4 and C_4 were gifts from Dr E. J. Goetzl, Harvard Medical School, Boston, Mass. ETYA was a gift from Dr W. Scott of Hoffman-La Roche, NDGA was obtained from Sigma, and BW 755C was a gift from Dr J. Vane, Wellcome Research Labs, Beckenham, Kent, U.K. Arachidonic acid was obtained from Sigma, and was kept for no longer than 1 month under nitrogen at −20°C.

Pituitary cell culture and LH release

Cultured rat pituitary cells were prepared by enzymatic dispersion with collagenase hyaluronidase [1] or trypsin [27] as previously described. The *in vitro* LH release assay was performed as previously described [1] with the modification that bovine serum albumin (BSA) was omitted from the incubation medium (medium 199 + 25 mM Hepes). Hormones and drugs were added to the dishes in 10 μl culture medium and incubations were performed for 3 h. The medium was then collected and saved at −20°C until assay for LH or other pituitary hormones content by radioimmunoassay using reagents provided by the NIAMDD pituitary Hormone Distribution Program. Results are expressed in terms of the RP-1 rat reference preparation as ng LH released per 10^5 cells. In specific experiments as noted, the actions of arachidonic acid, 5-HETE, and leukotriene B_4 were also examined in purified gonadotrophs prepared from trypsin-dispersed pituitary cells by centrifugal elutriation as described by Hyde *et al.*[27]. Prelabeling of cultured gonadotrophs with arachidonic acid was performed by incubation of culture wells containing 10^6 cells with 5 μCi of [^{14}C]arachidonic acid (50 mCi/mmol, Amersham) for 120 min at 37°C followed by extensive washing in medium 199. 0.1% BSA to remove free arachidonate. The cells were then exposed to stimuli (GnRH and A23187) for 2 h in medium 199, 0.1% BSA, and the medium was analyzed for release of [^{14}C]arachidonate.

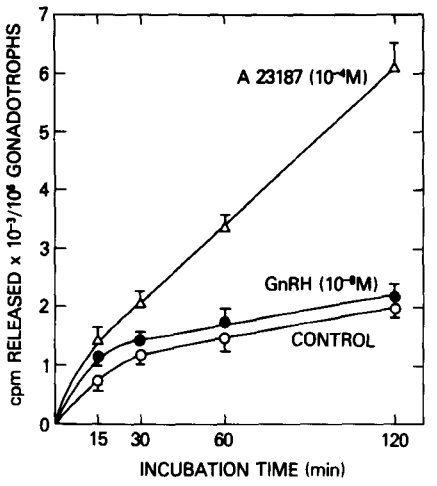


Fig. 1. Effects of GnRH and the calcium ionophore, A23187, on the release of [^{14}C]arachidonate from prelabeled pituitary gonadotrophs. Points are the mean ± SE of data from quadruplicate incubations.

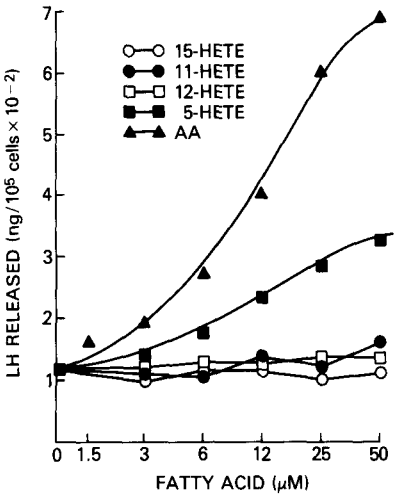
RESULTS

Stimulation of arachidonate release by GnRH and A23187

In purified gonadotrophs prelabeled with [^{14}C]arachidonic acid, exposure to the calcium ionophore. A23187, produced a marked increase in the rate of [^{14}C]arachidonate release (Fig. 1). In the same cell preparation, addition of 10^{-8} M GnRH caused a small but rapid and consistent increase in fatty acid release. This response was evident within 15 min and remained constant thereafter, in contrast to the progressive increase in response to the calcium ionophore for up to 120 min.

Actions of arachidonic acid and metabolites on LH release

When cultured pituitary cells were incubated with arachidonic acid or 5-HETE, dose–response curves for LH release were obtained with ED_{50} values of 10–15 μM. The dose–response curve for LH release in response to arachidonic acid was sometimes steeper than that elicited by 5-HETE (Fig. 2), but at higher concentrations (10^{-4} M) the two fatty acids caused similar responses of comparable magnitude to the stimulatory effect of 10^{-8} M GnRH. Of the other HETE compounds tested, 11-, 12-, and 15-HETE had no consistent effect on LH release at concentrations up to 50 μM (Fig. 3). At concentrations of 50 and 100 μM, 11- and 12-HETE were weak stimuli of LH release, though much less active than 5-HETE or arachidonic acid. Ricinoleic acid and methyl arachidonate had no significant effect on LH release (not shown). The ED_{50} value for arachidonic acid-induced LH release was about 10-fold lower than that reported previously, due to the omission of BSA from the incubation medium [15]. Cell viability as assessed by trypan blue exclusion was about 95% in cells treated with 1–50 μM arachidonic acid. Since concen-



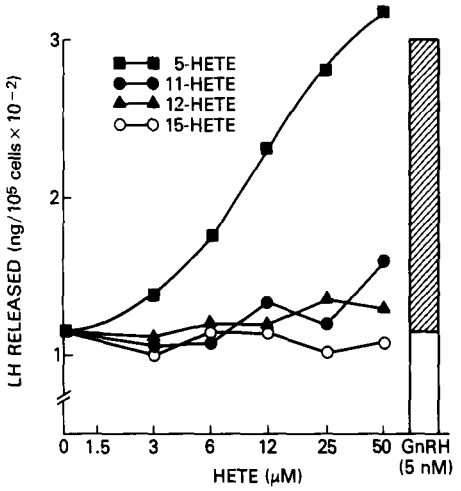


Fig. 2. Effects of arachidonic acid (AA) and HETE's on LH release. Three-day cultured pituitary cells (10^5/dish)] were incubated in medium 199/25 mM Hepes and various concentrations of fatty acids. Incubation was carried out for 3 h, and the medium was collected and saved for assay of LH. Above: LH responses to arachidonic acid and 5-HETE. Below: LH responses to 5-HETE and 5×10^{-9} M GnRH. All points in this and Figs 4 and 5 are the means of closely agreeing data ($\pm$ 10% SE) from triplicate incubations derived from at least three experiments.

trations of arachidonic acid above 50 μM (in the absence of BSA), or 125 μM (with BSA) increased cell permeability to trypan blue, the fatty acid was used only below these concentrations. The retention of functional integrity of pituitary cells cultured in the presence of arachidonic acid and then stimulated by GnRH is shown in Table 1, which demonstrates that LH responses to GnRH are unimpaired by prior exposure to the fatty acid. Arachidonic acid and 5-HETE, but not 11-, 12-, and 15-HETE, also caused a dose-dependent stimulation of prolactin and growth hormone release from cultured pituitary cells (not shown) indicating that the stimulatory effect is not restricted to the gonadotrophs.

Since 5-HPETE is converted to the leukotrienes, we also examined the effect of LTB_4 and LTC_4 on LH

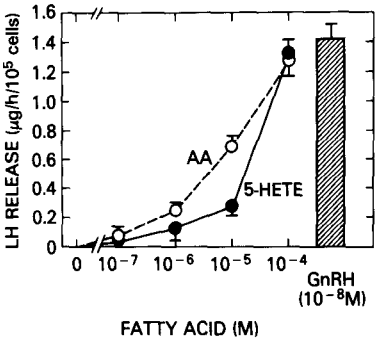


Fig. 3. Comparison of the concentration-dependent actions of arachidonic acid (AA) and 5-HETE on LH release in purified pituitary gonadotrophs. Data points in this study represent the mean $\pm$ SE of results from triplicate determinations.

release. These leukotrienes had only minor stimulatory effects on LH release at concentrations of up to 250 ng/ml or about 0.8 μM (Fig. 4). This range of concentrations was employed because leukotrienes have been reported to exert their biological effects as humoral mediators (slow reacting substance of anaphylaxis and chemotactic factor) in the 0.1 ng/ml range [28, 29]. Whereas the response to LTC_4 was apparent only at the lowest dose employed, that to LTB_4 was most evident at higher concentrations.

Effects of inhibitors of arachidonic acid metabolism on LH release

We have previously demonstrated that cyclooxygenase inhibitors (indomethacin or aspirin) have no inhibitory effect on GnRH-induced LH release [6,14,30]. In contrast, when incubated with cultured pituitary cells stimulated with 5 nM GnRH, the lipoxygenase inhibitors NDGA, ETYA, and BW 755C caused dose-dependent inhibition of LH release (Fig. 5). Half-maximal inhibition (IC_{50}) of GnRH-stimulated LH release was observed at 5, 8.5, and 175 μM with NDGA, ETYA and BW 755C, respectively. These drugs had no significant effect on basal LH release, indicating that their inhibitory actions on GnRH-stimulated hormone release are not related to a general impairment of pituitary cell function. On the other hand, when pituitary cells were incubated with the cyclooxygenase inhibitor,

Table 1. GnRH-stimulated LH release from cultured pituitary cells following exposure to arachidonic acid. LH release ($ng/10^5$ cells) is expressed as the mean $\pm$ SE of data from triplicate incubations

1st incubation	LH release	2nd incubation	LH release
Control	119 $\pm$ 9	Control	210 $\pm$ 14
		GnRH	568 $\pm$ 18
Arachidonic acid	242 $\pm$ 10	Control	141 $\pm$ 16
		GnRH	540 $\pm$ 12

Cultured pituitary cells (2.5×10^5) were initially incubated without or with 125 μM arachidonic acid for 2 h at 37°C and the media were collected for assay of LH. The cells were then washed and incubated in fresh medium without or with 10^{-8} M GnRH for an additional 2 h, and all media were assayed for LH content.

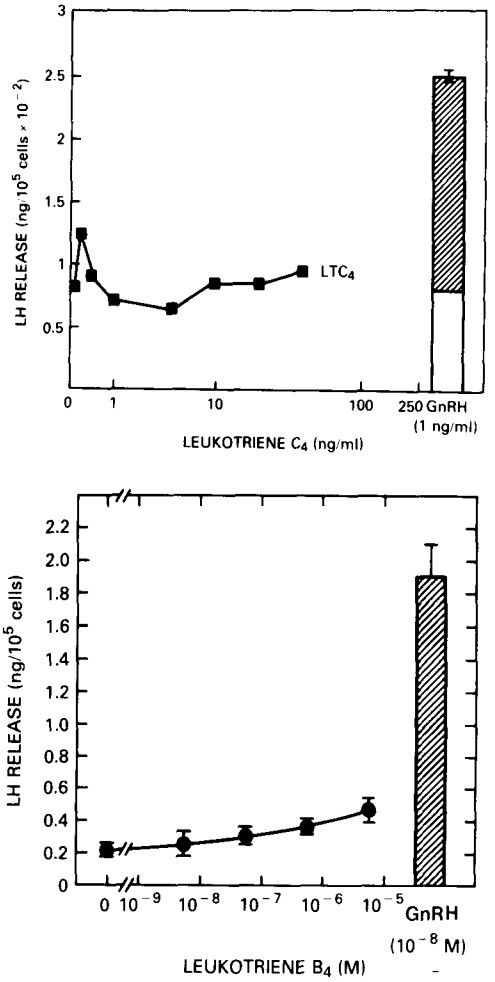


Fig. 4. Effects of leukotrienes B_4 and C_4 on LH release. Above: LH release in unfractionated pituitary cells incubated with increasing concentrations of LTC_4. Below: LH release in purified gonadotrophs incubated with LTE_4. In each case, the response of the cell preparation to GnRH is shown for comparison.

indomethacin, and a near-maximal concentration of GnRH (5 nM), there was consistently a biphasic effect on LH release. At concentrations up to 12–25 μM, indomethacin enhanced the LH response to GnRH by 40–50% of the control value, while at higher doses the drug had no effect on GnRH-induced hormone release.

DISCUSSION

We have recently reported that increased phospholipid turnover and the formation of free arachidonic acid might be involved in the action of GnRH agonists on pituitary gonadotropin release [14, 15]. Thus, GnRH causes an early increase in PI and PA turnover in cultured pituitary cells [14] that is accompanied by increased release of free AA into the culture medium [15]. The latter response was confirmed in the present study in purified gonadotrophs, which showed a rapid increase in fatty acid release when exposed to GnRH. Also, addition of arachidonic acid to cultured pituitary cells has been shown to elicit a marked increase in LH release [15]. The recent availability of arachidonic acid metabolites and lipoxygenase inhibitors has pemitted a more detailed analysis of the involvement of lipoxygenase products of arachidonic acid in GnRH action. Of the several products tested, arachidonic acid and 5-HETE were the most potent stimuli of LH release, with similar ED_{50} values for both fatty acids of about 12 μM. As previously observed, this value is about 10-fold lower for arachidonic acid than when BSA was present in the incubation medium [15], due to protein binding of the fatty acid. The stimulatory effect of arachidonic acid and 5-HETE in pituitary cells was not restricted to the LH producing cells, since we found a similar effect on the release of growth hormone and prolactin. This raises the possibility that increased phospholipid turnover and arachidonic acid production might reflect a general mechanism for pituitary hormone release.

The ability of 5-HETE to stimulate LH release suggests that the 5-lipoxygenase pathway could be involved in GnRH action. However, the LH response of cultured pituitary cells to 5-HETE was usually smaller than that elicited by arachidonic acid, except at the maximum effective concentration of about 100 μM fatty acid. If GnRH and arachidonic acid exert their stimulatory actions on LH release solely via production of 5-HETE, it might be expected that 5-HETE would be at least as active as arachidonic acid. It is also possible that 5-HETE regulates another lipoxygenase pathway, analogous to that described for 15-HETE [25], and that the other lipoxy-

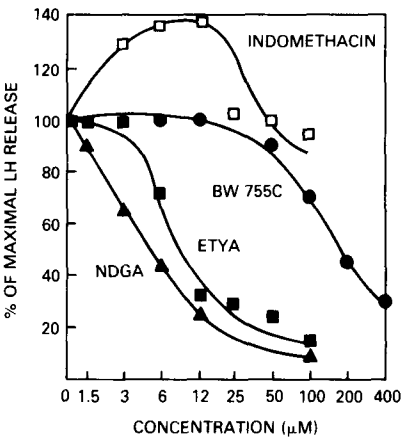


Fig. 5. Effects of lipoxygenase and cyclooxygenase inhibitors on GnRH-induced LH release. Cultured cells were initially incubated in medium 199/25 mM Hepes and various concentrations of the inhibitors for 30 min at 37°C. GnRH (5 nM) was then introduced in 10 μl of medium and incubation was continued for a further 3 h. Cells and medium were then separated and the medium assayed for LH content. Points represent the means of data from six experiments performed in triplicate. Basal and GnRH-induced LH levels were 75 ± 10 and 400 ± 30 ng/10^5 cells, respectively.

genase product is involved in GnRH action. 5-HETE is also known to act upon human neutrophils to release the granule enzyme lysozyme [31], and the stimulatory action of 5-HETE observed in this study might represent a more general effect of the hydroxy-fatty acid upon protein release from secretory granules. Another potential mechanism is that 5-HETE could act as a weak agonist analog of a more active metabolite of the 5-lipoxygenase pathway, such as 5-HPETE or leukotriene A_4. The ability of NDGA to block the arachidonate-induced release of LH also suggests that a lipoxygenase metabolite could be involved in the secretory mechanism. The leukotrienes are obvious candidates for such a role, but the minor effects of LTB_4 and LTC_4 on LH release under the present assay conditions are not indicative of a major action of these metabolites in gonadotropin secretion.

The lipoxygenase inhibitors, NDGA, ETYA and BW 755C, caused dose-dependent inhibition of GnRH-induced LH release. NDGA and ETYA inhibited GnRH action only in the absence of BSA from the incubation medium, possibly due to the binding of these drugs to albumin. The IC_{50} values obtained for NDGA and ETYA are in good agreement with those reported for depletion of cellular HETEs and suppression of chemotactic migration of neutrophils [27,28], and for inhibition of pituitary lipoxygenase activity (about 25 μM, Vanderhoek, Naor, and Catt, unpublished data). The differential selectivities of the inhibitors for the various lipoxygenase pathways, together with their differing potencies, are probably responsible for the diversity in IC_{50} values for NDGA and ETYA vs BW 755C [32, 33].

An interesting finding was the biphasic effect of the cyclooxygenase inhibitor indomethacin upon GnRH-induced LH release (Fig. 2). At low concentrations, indomethacin enhanced the effect of GnRH on LH release, and at higher concentration it had no effect. This biphasic effect of the drug could be related to the recent finding that at low concentrations the drug is a cyclooxygenase inhibitor, while at higher concentrations it also acts as a lipoxygenase inhibitor in neutrophils and platelets. In such studies, Siegel *et al.*[21] have observed that indomethacin concentrations up to 50 μM increase the total rate of conversion of arachidonic acid via the lipoxygenase pathway. The most likely explanation for the biphasic effect of indomethacin upon LH release is that low concentrations of the drug favor accumulation of HPETE products that are further metabolized via the lipoxygenase pathway, and that this action is lost at the higher concentrations (above 50 μM) at which indomethacin also acts as a lipoxygenase inhibitor [21]. The results obtained with indomethacin also indicate that arachidonic acid metabolites formed via the cyclooxygenase pathway are not involved in mediating GnRH-dependent LH release, in agreement with previous reports [6, 8, 14, 29]. On the other hand, our earlier observations [14] and the present findings strongly suggest that lipoxygenase metabolites exert an intermediate role in this process.

Leukotrienes and HETE compounds have been described in platelets, polymorphonuclear leukocytes, neutrophils, eosinophils and macrophages, and are believed to be involved in leukocyte chemotactic migration and immediate hypersensitivity [19–28, 31, 33]. A major implication of our study is that such compounds could be involved in the regulation of endocrine systems. By analogy, while the prostaglandins have been regarded as important humoral mediators of inflammation, they have also been recognized to act as ubiquitous modulators of target-cell functions.

Recently, isolated chromaffin granules aggregated by synexin, a calcium-binding protein of 47,000 Daltons [34], were shown to fuse together to form large secretory vesicles upon addition of *cis* unsaturated fatty acids, with release of part of their catecholamine stores [35]. Of the fatty acids tested, arachidonic acid was by far the most active fusogen. Since isolated chromaffin granules are regarded as a model for compound exocytosis [34], it was suggested that arachidonic acid might be involved in the process of exocytosis [35]. These observations are relevant to the findings of the present study, in which exocytosis of gonadotropins from intracellular granules was shown to be stimulated by arachidonic acid and its metabolites.

In considering the mechanism of action of GnRH on pituitary gonadotropin release, the time-course of the peptide's action should be taken into account. Recently, using a cell column superfusion technique to analyze the kinetic aspects of GnRH action [36], we observed that exposure of perifused pituitary cells to GnRH caused release of LH within 1 min, implying that the mechanism of action of GnRH should be manifested within the same time-scale. The action of thrombin upon human platelets has been shown to include increased formation of lysophosphatidylcholine, indicative of arachidonic acid release within 20 s of addition of the stimulus [37]. The kinetic properties of such ligand-induced responses emphasize the potential role of phospholipid turnover and arachidonic acid metabolites in the generation of "second messengers" during hormonal activation of such rapid responses as pituitary hormone release from preformed secretion granules. Further evidence for the general role of arachidonic acid and its metabolites in ligand-induced secretory processes has been provided by recent observations in the placenta [38], thyroid [39], and lung [40].

The present results would be consistent with a mechanism of action of GnRH on pituitary gonadotropin release that involves the following steps: after binding of GnRH to its specific, high-affinity receptors on the plasma membrane of pituitary gonadotrophs [1], activation of the GnRH receptors stimulates phospholipid turnover [30] and mobilizes calcium from both intracellular and extracellular

sources [2–5]. These processes possibly involve the participation of phosphatidic acid [30] and other potential calcium mobilizers such as inositol triphosphate [16] that are formed during phosphoinositide breakdown. Calcium mobilization is accompanied by activation of phospholipase A_2 and/or diglyceride lipase with formation of free arachidonic acid [14]. The newly-released arachidonic acid, or one of its lipoxygenase or epoxygenase [41] products, causes release of stored gonadotropins by promoting granule fusion with the cell membrane. Independent of any change is calcium mobilization, diacylglycerol, the other major metabolite formed during phosphoinositide metabolism, can activate protein kinase C [16], an enzyme that appears to be involved in the action of GnRH on gonadotrophin release [42]. The increased turnover of phospholipids and fatty acids involved in GnRH action also stimulates guanylate cyclase and increases cellular levels of cyclic GMP [13, 14, 43]. Associated changes in cyclic AMP production are sometimes observed [44], but increased formation of cyclic nucleotides does not appear to be a prerequisite for the acute effect of GnRH on LH release [1, 7–13]. While changes in cyclic AMP might be involved in gonadotropin synthesis and long-term responses to the decapeptide [45, 46], the role of cGMP in GnRH action is not yet clearly defined.

In summary, the present study has provided further evidence for the involvement of lipoxygenase products of arachidonic acid in the mechanism of action of GnRH on LH release. Although the actions of GnRH on gonadotropin release could also be mediated by an as yet unidentified metabolite of arachidonic acid, it seems more likely that a product of the 5-lipoxygenase pathway acts as an intermediate in the release process. Such an effect would be consistent with the present observations on the effects of arachidonic acid and lipoxygenase inhibitors on LH release. Further studies are in progress to identify the active intermediary products of arachidonic acid that are involved in the peptide's action on LH release in purified rat gonadotrophs.

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