

Delayed activation of phospholipase D by gonadotropin-releasing hormone in a clonal pituitary gonadotrope cell line (α T3-1)

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Stimulation of cultured pituitary cells from a gonadotrope lineage (α T3-1) by the gonadotropin-releasing hormone agonist analog [D-Trp⁶]GnRH (GnRH-A) resulted in a manifold increase in accumulation of phosphatidylethanol, a specific product of phospholipase D phosphatidyl transferase activity when ethanol is the phosphatidyl group acceptor. Levels of the natural lipid product of phospholipase D, phosphatidic acid, were increased 2–3-fold. Activation of phospholipase D by GnRH-A was dose- and time-dependent and was blocked by a GnRH receptor antagonist [D-pClPhe², D-Trp^{3,6}]GnRH. GnRH-A stimulated phospholipase D activity after a lag of 1–2 min. We conclude that in α T3-1 gonadotropes GnRH receptor occupancy results in delayed activation of phospholipase D which could participate in late phases of gonadotrope regulation by the neurohormone.

Diacylglycerol; GnRH; Phosphatidylcholine; Phosphatidic acid; Protein kinase C; Signal transduction

1. INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is the first key hormone of the reproductive system. Nevertheless, its mechanism of action is not fully understood [1]. Previous studies have shown that GnRH stimulates a phosphoinositide-specific phospholipase C activity [2,3], mobilizes cellular and extracellular Ca^{2+} [4–6], activates protein kinase C [7,8] and induces the release of arachidonic acid [9]. More recently it was shown that a GnRH agonist analog stimulates phospholipase D activity in cultured rat granulosa cells, and evidence implicating phospholipase D activation in signaling granulosa cell differentiation was presented [10]. Yet, the role of phospholipase D in GnRH action on its classical target cells, the pituitary gonadotropes, remained unexplored. The recent recognition of the potential importance of phospholipase D in signal transduction [11] prompted us to examine the effect of GnRH upon phospholipase D activity in cultured pituitary cells. Pituitary cells are heterogeneous and only about 10% are GnRH-responsive cells (gonadotropes). We have therefore utilized here a clonal pituitary cell line of the gonadotrope lineage α T3-1 which was obtained by targeted oncogenesis in transgenic mice [12].

2. EXPERIMENTAL

The α T3-1 cell line was kindly provided by Dr. P. Mellon (Salk

Institute, La Jolla, CA) and was cultured as described by Mellon et al. [12,13]. To preferentially label the phosphatidyl moiety of phosphatidylcholine, cells ($1.5\text{--}3 \times 10^5/35\text{-mm}$ dish) were routinely preincubated overnight (16–24 h) with [³H]myristic acid (5 $\mu\text{Ci/ml}$ /dish) in DMEM containing fatty acid-free bovine serum albumin (BSA; 0.18%). Stimulants were then added in 100 μl of the above medium in the presence or absence of ethanol (0.5%) for the indicated time intervals and incubations were terminated by placing the dishes on ice and removal of medium. Cold methanol (1 ml) was added and the cells were scraped and transferred to 12 \times 75-mm glass tubes. Chloroform (1 ml) and a mixture of 0.1 N HCl, 1 mM EGTA (1 ml) were added. The tubes were vigorously mixed, centrifuged (2000 rpm, 10 min) and the lower chloroform phase was collected and dried under vacuum. [³H]Phosphatidic acid and [³H]phosphatidylethanol were separated by thin layer chromatography on oxalate-impregnated Whatman LK6 plates developed with the organic phase of a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10) as previously described [10,14]. The regions corresponding to the appropriate standards were scraped, extracted with 1 ml of methanol/HCl (150:1) and counted after addition of 9 ml of Insta-Mix (Packard). [9,10-³H(N)]-Myristic acid (33.9 Ci/mmol) was purchased from Dupont-New England Nuclear (Boston, MA). GnRH agonist and antagonist were kindly provided by Dr. D. Coy (Tulane University, LA).

3. RESULTS AND DISCUSSION

In a preliminary study we examined the effect of GnRH and GnRH-A on phospholipase D activity in cultured, primary rat pituitary cells. While GnRH-A caused some stimulation of phospholipase D in most experiments, the response was weak, most likely due to the fact that GnRH-responsive cells (gonadotropes) constitute only about 10% of the total pituitary cell population.

We next examined the effect of GnRH-A on phospholipase D activity in the clonal α T3-1 cell line which

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is of gonadotrope lineage and was derived by targeted oncogenesis in transgenic mice [12]. GnRH binds with high-affinity to specific binding sites in α T3-1 cells and stimulates phosphoinositide-specific phospholipase C activity within 30 s of incubation [13]. GnRH-A stimulated the accumulation of α T3-1 cells of both the natural lipid product of phospholipase D, phosphatidic acid and, in the presence of ethanol, of the specific phospholipase D product, phosphatidylethanol, in a dose-dependent manner (Fig. 1). EC_{50} values for [3 H]phosphatidic acid and [3 H]phosphatidylethanol formation were 0.25 and 10 nM, respectively. These values are comparable to the K_d of GnRH-A binding to the GnRH receptor (ca. 3 nM; [15]) but are much higher than the EC_{50} value for LH release (ca. 0.03 nM; [15]). The results imply that activation of phospholipase D is tightly coupled to receptor occupancy, while LH release requires occupancy of only a fraction of the receptors (ca. 20%; [16]). Interestingly, whereas maximal formation of [3 H]phosphatidic acid (achieved at 10 nM of GnRH-A) was followed by a decrease at higher concentrations, there was no comparable decrease in formation of [3 H]phosphatidylethanol at concentrations above 10 nM of GnRH-A (Fig. 1). Similar results were obtained in GnRH-A-stimulated granulosa cells [10]. The results suggest the interesting possibility that, at high concentrations, GnRH stimulates the activity of a signal termination pathway, possibly involving phosphatidic acid degradation into diacylglycerol or lysophosphatidic acid.

The stimulatory effect of GnRH-A upon phospholipase D activity is a receptor-mediated response as co-

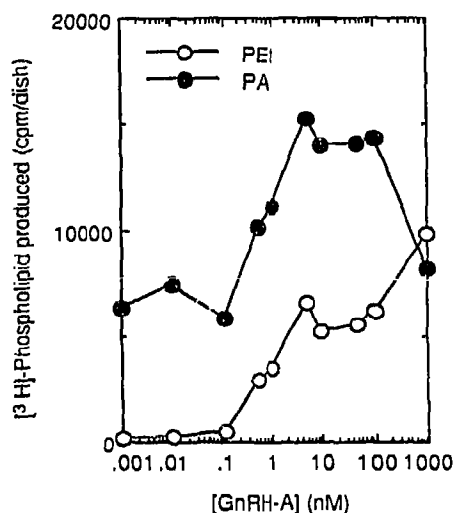


Fig. 1. Stimulation of phospholipase D activity by GnRH-A in α T3-1 gonadotropes. Cells were prelabeled with [3 H]myristic acid and incubated for 60 min with the indicated concentrations of the GnRH agonist analog [D-Trp^{3,6}]GnRH. Incubations were terminated, lipids were extracted and [3 H]phosphatidic acid (PA) and [3 H]phosphatidylethanol (PEt) were quantitated as detailed in section 2. Results are the mean of duplicate determinations from a representative experiment.

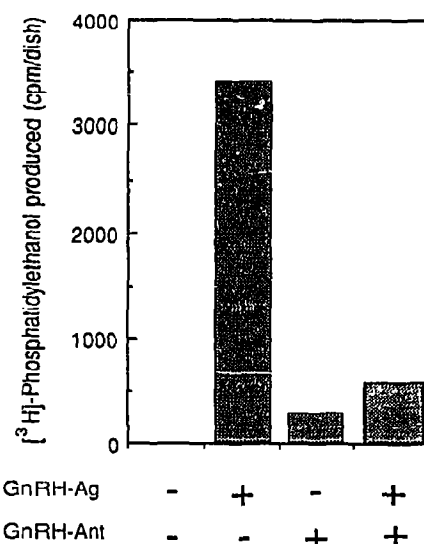


Fig. 2. Inhibition of GnRH-A-induced activation of phospholipase D by a specific GnRH receptor antagonist. Cells were prelabeled with [3 H]myristic acid and incubated for 60 min with GnRH-A (1 nM) alone, or together with 100 nM of the GnRH antagonist analog [D-pClPhe², D-Trp^{3,6}]GnRH (GnRH-Ant) as indicated. Incubations were terminated, lipids were extracted and [3 H]phosphatidylethanol was quantitated as detailed in section 2. Results are the mean of duplicate determinations from a representative experiment.

incubation with a potent GnRH receptor antagonist [D-pClPhe², D-Trp^{3,6}]GnRH nearly abolished the response (Fig. 2). The time-course of the effect of GnRH-A on phospholipase D activity is shown in Fig. 3. There was a lag of 1–2 min before detectable accumulation of

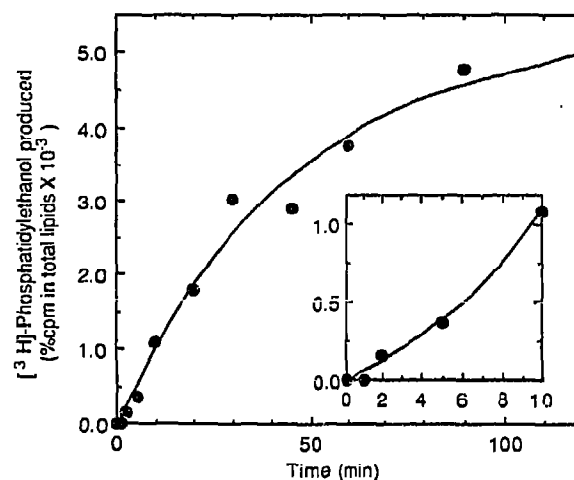


Fig. 3. Time-course of [3 H]phosphatidylethanol accumulation in GnRH-A-stimulated α T3-1 gonadotropes. Cells were prelabeled with [3 H]myristic acid and incubated for the indicated time with GnRH-A (10 nM). Incubations were terminated, lipids were extracted and [3 H]phosphatidylethanol was quantitated as detailed in section 2. Results are expressed as the percentage of cpm incorporated into total lipids and are the mean of duplicate determinations from a representative experiment. The inset shows a detailed view of the first 10 min of the incubation.

[^3H]phosphatidylethanol could be observed. Maximal rates of PLD activity were observed between 10 and 60 min of incubation, and no further increase of [^3H]phosphatidylethanol accumulation could be observed between 90 and 120 min. The delayed activation of phospholipase D by GnRH-A in $\alpha\text{T3-1}$ cells suggest that the stimulation of phospholipase D activity is not a primary response to receptor stimulation.

Stimulation of gonadotropin secretion and gene expression by GnRH is a complex signaling process involving phosphoinositide-specific phospholipase C, Ca^{2+} , protein kinase C and arachidonate and its products (see [1] for review). In addition, gonadotropin secretion induced by GnRH is a biphasic process which might require diacylglycerol in an immediate as well as a delayed kinetics for biphasic activation of protein kinase C [17–19]. As phosphatidylinositol 4,5-bisphosphate and even phosphatidylinositol are minor phospholipids as compared to phosphatidylcholine, generation of diacylglycerol from phosphatidylinositol and phosphatidylinositol 4,5-bisphosphate (via phospholipase C) might represent an initial rapid response, while the bulk of diacylglycerol might be derived from phosphatidylcholine via phospholipase D to serve as a protein kinase C activator during the sustained phase of hormone action. Since protein kinase C is implicated also in gonadotropin gene expression (see [1] for review), delayed supply of diacylglycerol (via phospholipase D) might distinguish between immediate and sustained responses to the neurohormone. Indeed, kinetic analysis reveals that while GnRH-stimulated phospholipase C activity is detected within 5–10 s in normal pituitary cells [2] or within 30 s in $\alpha\text{T3-1}$ cells [13], stimulation of phospholipase D activity was observed in this study only after a lag of 1–2 min. Thus, activation of phospholipase D by GnRH in $\alpha\text{T3-1}$ cells follows the activation of phospholipase C, and might be dependent on this initial response. Both Ca^{2+} and protein kinase C were reported to be involved in phospholipase D activation [20–22], although the extent of their involvement may vary widely in different cell types (cf. [10,23]). It is therefore possible that initially, GnRH activates phospholipase C to generate a transient rise of Ca^{2+} and a limited amount of diacylglycerol, and consequently, a rapid activation of protein kinase C. This would lead to stimulation of phospholipase D activity which might generate a second phase of diacylglycerol for sustained activation of protein kinase C. Further studies are required in order to fully evaluate this proposed sequence of signaling.

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