Calcium-independent phosphatidylinositol response in gonadotropin-releasing-hormone-stimulated pituitary cells

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This paper describes the effect of gonadotropin-releasing hormone (GnRH, gonadoliberin) on phospholipid metabolism in cultured rat pituitary cells. The cells were incubated with [^{32} P]P_i to label endogenous phospholipids (10–60 min) and then stimulated with GnRH for up to 60 min. Cellular phospholipids were separated by two-dimensional t.l.c. and the radioactivity was determined. Phosphatidylinositol (PI), a minor constituent of cellular phospholipids (7.7%), was the major labelled phospholipid, accounting for 45% of the total radioactivity, at early periods after pulse labelling. On the other hand, phosphatidylcholine, the major cellular phospholipid (37%), was labelled only to 32% of the total radioactivity. The remaining label was distributed among phosphatidylethanolamine (4.2%), cardiolipin (3.4%), phosphatidic acid (PA, 2.5%), and phosphatidylserine (1.8%). GnRH doubled ^{32} P labelling of PA and PI significantly at 1 and 5 min of incubation respectively in the presence or absence of extracellular Ca²⁺. Labelling of other phospholipids was not affected by GnRH treatment. The half-maximal stimulating dose (ED₅₀) for PI labelling and lutropin release was 0.75 nM and 0.5 nM respectively, and the stimulatory effect was blocked by the potent GnRH antagonist [D-Glp¹, pCIPhe², D-Trp^{3,6}]GnRH. GnRH-stimulated PA and PI labelling could not be demonstrated after 1 and 45 min of incubation respectively, or when the prelabelling was conducted for 60 min rather than 10 min. These results suggest heterogeneous compartmentalization of gonadotroph PA and PI pools and that increased PI turnover might be a transducing signal for Ca²⁺ gating that follows gonadotroph GnRH-receptor activation.

INTRODUCTION

The mechanism of action of GnRH on pituitary lutropin (luteinizing hormone) and follitropin (follicle-stimulating hormone) biosynthesis and release has not yet been elucidated. Neither prostaglandins (Naor *et al.*, 1975a) nor cyclic AMP or cyclic GMP (Naor *et al.*, 1975b; Sundberg *et al.*, 1976; Conn *et al.*, 1979; Naor & Catt, 1980; Naor, 1982) were found to act as mediators ('second messengers') of GnRH-induced gonadotropin release. On the other hand, an essential role of extracellular Ca²⁺ has been demonstrated (Samli & Geschwind, 1968; Wakabayashi *et al.*, 1969; Kraicer, 1975; Naor *et al.*, 1980b; Conn *et al.*, 1981). More recently, we and others have suggested that phospholipid turnover and lipoxygenase and/or epoxigenase derivatives of arachidonic acid might be involved in GnRH action on gonadotropin release (Naor & Catt, 1981; Naor *et al.*, 1983; Snyder *et al.*, 1983). It is well recognized that one of the earliest biochemical events involved in receptor-mediated phospholipid turnover is the phosphatidylinositol (PI) response, first observed by Hokin & Hokin (1953), and later suggested as a more general mechanism for ligands which do not operate via cyclic AMP production (Michell, 1975; Michell *et al.*, 1981; Berridge, 1982, 1984).

We and others previously demonstrated that GnRH analogues increase PI and PA labelling, in cultured ovarian granulosa and luteal cells (Naor & Yavin, 1982; Davis *et al.*, 1983; Leung *et al.*, 1983) and the labelling

of PI in dispersed testicular interstitial cells (Molcho *et al.*, 1984a). Since the mechanism of action of GnRH in the pituitary has still not been elucidated, it was decided to evaluate whether PI and PA labelling is also increased by GnRH in pituitary gonadotrophs, and the Ca²⁺-sensitivity of this response.

Preliminary studies have presented different results regarding GnRH-stimulated PI and PA labelling in the pituitary (Snyder & Bleasdale, 1982; Raymond *et al.*, 1984). We decided to analyse the early effects of GnRH on cultured pituitary cells, using ^{32} P as a probe for phospholipid labelling, and to compare the labelling with that of gonadal cells, where GnRH analogues were previously shown to stimulate PI labelling (Naor & Yavin, 1982; Davis *et al.*, 1983; Leung *et al.*, 1983; Molcho *et al.*, 1984a). The role of Ca²⁺ in GnRH-induced PI labelling was also investigated.

EXPERIMENTAL

Cell preparation

Pituitary glands of 12 Wistar-derived rats (200–250 g body wt.) from the Departmental colony, 40–60 days after ovariectomy, were used for cell preparation by a modification of the technique described by Hopkins & Farquhar (1973). Pituitaries were cut finely and incubated consecutively at 37 °C in 5 ml volumes of medium 199 with 0.3% bovine serum albumin, containing: first, 5 mg of trypsin (Sigma)/ml for 15 min, followed by 2.5 µg of

Abbreviations used: GnRH, gonadotropin-releasing hormone (gonadoliberin); PI, phosphatidylinositol; PA, phosphatidic acid.

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deoxyribonuclease (Sigma)/ml for 1 min, and thereafter, 1 mg of soya-bean trypsin inhibitor (Sigma)/ml for 5 min. Then two additional incubations were performed in Ca^{2+} - and Mg^{2+} -free Dulbecco's phosphate buffer with 0.3% bovine serum albumin, and containing, respectively, 2 mM-EDTA (5 min) and 1 mM-EDTA (15 min). The tissue blocks were then transferred to a 17 mm $\times$ 100 mm polycarbonate tube, washed in Ca^{2+} - and Mg^{2+} -free Dulbecco's phosphate buffer, and dispersed into single cells by gentle aspiration and extrusion through a plastic Pasteur pipette in 5 ml of the same buffer. The cell suspension was filtered through nylon gauze and diluted in medium 199 with 10% (v/v) horse serum, penicillin (100 units/ml) and streptomycin (100 μg /ml). Cell yield was 3×10^6 – 5×10^6 cells per pituitary (80–90% viability). Batches of cells were then applied (4×10^6 cells/well) to tissue-culture plates (35 mm diam.; Falcon) and maintained in culture for 2 days in a humidified incubator. We took advantage of the fact that cells derived from pituitaries of ovariectomized rats (40–60 days after ovariectomy) contain about 26% of gonadotrophs, as compared with 12% of gonadotrophs (lutropin + follitropin cells) in normal pituitary gland of a female rat (G. Childs, personal communication). For comparative studies, ovarian granulosa cells were prepared as described by Naor & Yavin (1982) and analysed after 2 days in culture. Testicular interstitial cells were prepared from adult male rats as described by Molcho *et al.* (1984a, b) and analysed on the same day.

Phospholipid labelling

The culture medium was removed and replaced by phosphate-free Krebs-Ringer bicarbonate buffer (KRB), pH 7.4, containing glucose (1 mg/ml). Endogenous phospholipids were labelled for 10–60 min at 37 °C by adding 50 μCi of [^{32}P]P_i (carrier-free; 10 mCi/ml; Amersham, Arlington Heights, IL, U.S.A.) per dish. GnRH (Peninsula Laboratories, San Carlos, CA, U.S.A.) or a GnRH antagonist [D-Glp¹, pClPhe², D-Trp^{3,6}]GnRH (kindly supplied by Dr. D. Coy, Tulane University, New Orleans, LA, U.S.A.) were then introduced, and incubation was continued for up to 60 min. To investigate the role of Ca^{2+} , cells were incubated consecutively at 37 °C in KRB (containing 1.2 mM- Ca^{2+}) containing [^{32}P]P_i for 10 min, followed by incubation in

KRB- Ca^{2+} + EGTA (2 mM) for 10 min, and finally in KRB- Ca^{2+} + EGTA (1 mM) + GnRH (100 nM) for 10 min. Cells were harvested in methanol and transferred to Eppendorf conical tubes. Cell phospholipids were then extracted with chloroform/methanol (2:1, v/v), followed by sonication for 1 min (Branson sonifier B-12). After centrifugation (20000 *g* for 10 min), the supernatant was collected, evaporated under N₂, and redissolved in 0.5 ml of chloroform. Samples were washed with 0.5 ml of chloroform/methanol/water (8:4:3, by vol.), and the lower phase was collected, dried, and redissolved in 25 μl of chloroform/methanol (2:1, v/v). Samples were then applied to silica-gel-G-coated plates (Kieselgel 60; Merck, Darmstadt, West Germany), and phospholipids were identified by two-dimensional t.l.c. with chloroform/methanol/methylamine (13:7:3, by vol.) for the first direction, followed by a second separation with chloroform/acetone/methanol/acetic acid/water (10:4:2:3:1, by vol.), as described by Yavin & Zutra (1977). The plates were exposed to autoradiography (AGFA RP2 film), the radioactive spots were identified with markers, and the corresponding areas of the various phospholipids were removed and counted for radioactivity by the Čerenkov technique. The inorganic phosphorus contents of total lipid extract and of individual phospholipids were determined by the method of Bartlett (1959). For statistical analyses, data were pooled from several experiments, and the means were compared with control values by Student's *t* test.

RESULTS

Several methods of phospholipid separation were examined, and the two-dimensional separation described by Yavin & Zutra (1977) was found to be the most appropriate to ensure a complete separation of PI and PA from other labelled phospholipids. Several ^{32}P -labelled phospholipid species were extracted and separated from prelabelled cultured pituitary cells (Table 1). We have compared the results with those obtained from ovarian granulosa cells and testicular interstitial cells, in which it was reported that GnRH agonists increase PI labelling (Naor & Yavin, 1982; Davis *et al.*, 1983; Leung *et al.*, 1983; Molcho *et al.*, 1984a). Interestingly, labelling of phosphatidylserine, phosphatidylglycerol and cardiolipin

Table 1. Phospholipid labelling and mass ratio of various phospholipids in GnRH target organs

Cultured pituitary cells, ovarian granulosa cells and testicular interstitial cells (4×10^6 /dish) were incubated with [^{32}P]P_i for 60 min, and the various phospholipids were separated and the radioactivity and inorganic phosphorus were determined (*n* = 6). Abbreviations: N.D., not determined; U.D., undetectable; % Rad, % of radioactivity.

Phospholipid	Pituitary		Ovary		Testis	
	% Rad	% Mass	% Rad	% Mass	% Rad	% Mass
Phosphatidylinositol (PI)	45	7.7	6.5	6.0	9.6	9.0
Phosphatidic acid (PA)	2.5	U.D.	1.75	U.D.	6.6	U.D.
Phosphatidylserine	1.8	7.5	2.0	9.8	U.D.	7.0
Phosphatidylethanolamine	4.5	16.7	3.5	19.8	3.3	17.4
Ethanolamine plasmalogen	3.3	13.3	2.5	12.8	2.5	13.0
Cardiolipin	3.4	N.D.	2.0	N.D.	U.D.	N.D.
Phosphatidylglycerol	N.D.	N.D.	2.0	N.D.	0.50	N.D.
Phosphatidylcholine	32	37	64	33	62	35
Choline plasmalogen	9.0	8.8	8.5	8.7	8.5	9.7
Lysophosphatidylcholine	N.D.	N.D.	4.0	N.D.	10	N.D.

was observed in pituitary and ovarian cells, but not in testicular interstitial cells.

PI, a relatively minor constituent of cellular phospholipids (7.7%), was labelled up to 45% (of total radioactivity incorporated into the phospholipid fraction) after 60 min of labelling of cultured pituitary cells (Table 1). On the other hand, phosphatidylcholine, a major phospholipid (37% of mass), was labelled only to 32% of the total. The opposite was observed in gonadal cells; the majority of the radioactivity was incorporated into phosphatidylcholine (63%), whereas PI labelling was low and approached an isotopic equilibrium to match its mass distribution closely (6–10%). Thereafter (2–3 h) the relative distribution of label among pituitary phospholipids resembles the gonadal distribution (results not shown). This is expected, since, in terms of mass, the distribution of the major phospholipid species in pituitary and gonadal cells is very similar (Table 1).

In the course of this study, several ^{32}P -labelling schedules were tested to optimize the effect of GnRH on PI and PA labelling. When cultured pituitary cells were prelabelled for 10 min with ^{32}P and later stimulated with a maximal dose of GnRH (100 nM), increased PA and PI labelling was observed (Fig. 1). Significant

Fig. 1. Time-response study of the effect of GnRH on PI (a) and PA (b), labelling in cultured pituitary cells

The cells were prelabelled for 10 min with ^{32}P and later incubated with GnRH (100 nM) for the time indicated. Each point is the mean $\pm$ S.E.M. for six determinations from three different experiments. (a) PI labelling: $\blacksquare$, control; $\blacktriangle$, +GnRH. (b) PA labelling: $\square$, control; $\hatched$, +GnRH.

Fig. 2. Dose-response study for GnRH-induced PI labelling in cultured pituitary cells

The cells were prelabelled for 10 min and later stimulated with GnRH for 10 min. Each point is the mean $\pm$ S.E.M. for six determinations ($\text{ED}_{50} = 0.75$ nM).

stimulation of PA labelling by GnRH was observed within 1 min of incubation ($P < 0.02$), whereas GnRH-stimulated PI labelling exhibited a lag time of 5 min before a significant increase could be demonstrated ($P < 0.05$). GnRH did not elicit a response on PA and PI labelling after 1 and 45 min incubation respectively, or when the prelabelling was carried on for 60 min or more rather than 10 min as described in Fig. 1. Labelling of other phospholipids, such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, was not significantly affected by the GnRH treatment.

The dose-response study for GnRH stimulation of PI labelling is shown in Fig. 2. Maximal stimulation was obtained when the experiment was carried out for 10 min, after 10 min of prelabelling. The amount giving half-maximal stimulation (ED_{50}) of PI labelling was observed to be 0.75 nM-GnRH, which is in good agreement with the ED_{50} for stimulation of lutropin release from cultured gonadotrophs (0.5 nM; Naor *et al.*, 1980a).

The role of Ca^{2+} in GnRH-induced PI turnover was investigated (Fig. 3). GnRH increased ^{32}P labelling of PI in the presence or absence of extracellular Ca^{2+} , but stimulated lutropin release only in the presence of extracellular Ca^{2+} (Naor *et al.*, 1980b). Interestingly, removal of Ca^{2+} increased PI labelling in the unstimulated cells by 68% (results not shown), most likely owing to inhibition of PI resynthesis by Ca^{2+} . Indeed, CDP-diacylglycerol-inositol phosphatidyltransferase is inhibited by Ca^{2+} (Egawa *et al.*, 1981).

To demonstrate specificity, a potent antagonist, $[\text{D-Glu}^1, \text{pClPhe}^2, \text{D-Trp}^3, 6]\text{GnRH}$, was employed (Fig. 4). GnRH (100 nM) increased the specific labelling of PI by 300%, and the antagonist (1 μM) nearly abolished the stimulatory effect of GnRH on PI labelling.

DISCUSSION

The present study demonstrates an early stimulatory effect of GnRH on PA and PI labelling in cultured gonadotrophs, which is not dependent on extracellular Ca^{2+} . Thus PI turnover might mediate GnRH-induced Ca^{2+} gating, as was suggested for other ligands which did not act via cyclic nucleotide production (Michell, 1975; Michell *et al.*, 1981; Berridge, 1982, 1984).

Fig. 3. Ca²⁺-sensitivity of GnRH-induced PI labelling

Cells were prelabelled for 10 min in KRB containing [³²P]P_i, followed by incubation in KRB or in KRB-Ca²⁺+EGTA (2 mM) for 10 min. Medium was then changed to KRB or KRB-Ca²⁺+EGTA (1 mM), and GnRH (100 nM) was added in 10 μ l. Incubation was for 10 min and the bars represent the means $\pm$ S.E.M. for six determinations from three different experiments.

Fig. 4. Effect of GnRH antagonist on GnRH-induced PI labelling

Cultured pituitary cells were prelabelled for 10 min and later incubated with the potent antagonist [D-Glp¹, pClPhe², D-Trp^{3,6}]GnRH (1 μ M) and GnRH (100 nM) for 15 min. The bars represent the means $\pm$ S.E.M. for six determinations from three different experiments.

The data reported here indicate that GnRH markedly and rapidly increases the specific labelling of PI and PA in cultured gonadotrophs in the absence of increased Ca²⁺. The effect is remarkable in that PI and PA represent only a minor fraction of cellular phospholipid content (both together about 10% of total mass). Nevertheless, in short-term incubation periods pituitary PI labelling

from ³²P precursor represents 45% of total radioactivity incorporated into the PL fraction, whereas phosphatidylcholine labelling amounts to only 32% of total. In contrast, GnRH-responsive gonadal cells contain less than 10% of labelled PI and 60% of labelled phosphatidylcholine (Table 1). The pool size of PI in pituitary and gonadal cells is nearly the same (6–9%), yet the turnover in the pituitary is much faster than that of the ovarian or testicular PI. Whether this observation has to do with the relatively rapid kinetics of pituitary function needs to be determined.

It is noteworthy that GnRH stimulation of PA and PI labelling could be detected only up to 1 and 45 min of incubation respectively and only when the prelabelling period was 10 min, but not 60 min. It is possible therefore that pituitary gonadotrophs contain a minor fraction of a GnRH-sensitive PA and PI pool with a high turnover rate and a larger pool of hormone-insensitive PA and PI with lower turnover rate. In short incubation periods the hormone-sensitive pool is labelled, and the increased labelling can be detected. On the other hand, the longer incubation periods might also label the hormone-insensitive pool and thereby mask the stimulatory effect of GnRH. A hormone-sensitive pool of PI was reported in WRK-1 cells by Monaco & Woods (1983).

In most cell systems in which agonist-stimulated PI turnover is associated with Ca²⁺ mobilization, the effect occurs independently of extracellular Ca²⁺ (Michell *et al.*, 1981; Berridge, 1982). The ability to demonstrate enhanced PI turnover in the absence of Ca²⁺ and in the presence of EGTA will therefore be consistent with the hypothesis that increased PI turnover is part of the mechanism by which an increase in Ca²⁺ is generated. However, in other systems (e.g. rabbit neutrophils; Cockcroft *et al.*, 1980), ligand-stimulated PI turnover occurs only in the presence of Ca²⁺. In our system, GnRH stimulated PI response in the absence of extracellular Ca²⁺ and the presence of EGTA. Nevertheless, Ca²⁺ mobilization via a receptor-independent mechanism such as ionophore A23187 can also affect the PI response via a different mechanism (for review see Berridge, 1982; Michell *et al.*, 1981). The observation that GnRH-induced PI labelling occurred in the absence of extracellular Ca²⁺, whereas stimulated lutropin release was completely blocked (Naor *et al.*, 1980b), supports the postulate that PI turnover is involved in Ca²⁺ gating (Michell, 1975; Salmon & Honeyman, 1980; Putney *et al.*, 1980; Michell *et al.*, 1981; Berridge, 1982, 1984), which is later responsible for gonadotropin release.

In terms of the time course of GnRH action, the effect reported here is in good agreement with the rapid effect of GnRH on lutropin release (approx. 1 min; Naor *et al.*, 1982). The ED₅₀ for GnRH-stimulated PI turnover (0.75 nM) and lutropin release (0.5 nM; Naor *et al.*, 1980a) are in close agreement, lending support to the proposal that the PI response is involved in GnRH action on lutropin release. Whether an early event in GnRH action includes polyphosphoinositide degradation (Michell *et al.*, 1981; Berridge, 1984), as suggested for thyrotropin-releasing hormone (thyroliberin) (Martin, 1983; Rebecchi & Gershengorn, 1983), and the release of endogenous bound Ca²⁺ by inositol 1,4,5-trisphosphate (Streb *et al.*, 1983) is not clear at the present time and needs further investigation in purified gonadotrophs.

The results reported here suggest that the PI response might serve as a transducing signal for Ca²⁺ gating in

GnRH action in the pituitary, and thus present an alternative mechanism of action for peptide hormones which do not act via cyclic nucleotide production.

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