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Does oligosaccharide-phosphatidylinositol (glycosyl-phosphatidylinositol) hydrolysis mediate prolactin signal transduction in granulosa cells?

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Initial biosynthetic radiolabelling experiments with cultured granulosa cells revealed the presence of an oligosaccharide-phosphatidylinositol (glycosyl-phosphatidylinositol; $(Ose)_n$PtdIns) structurally related to $(Ose)_n$PtdIns-lipids isolated from other cell types. Prolactin (PRL) stimulated [^{3}H]glucosamine-$(Ose)_n$PtdIns turnover and the rapid generation of [^{3}H]myristoyl-diacylglycerol in cultured follicle-stimulating hormone-(FSH)-primed granulosa cells endowed with PRL receptors. In parallel experiments performed with [^{3}H]*myo*-inositol-labelled granulosa cells, treatment with PRL stimulated $(Ose)_n$PtdIns hydrolysis in a similar manner, whereas no effect on phosphoinositide (PtdIns, PtdIns*P* and PtdInsP_2) turnover could be observed. These results strongly suggest that the cleavage of $(Ose)_n$PtdIns by phosphodiesterase followed by the subsequent generation of diacylglycerol and a soluble phosphoinositol-oligosaccharide (inositol-phosphoglycan; $(Ose)_n$Ins*P*) moiety could be part of the signal-transduction mechanism linking PRL receptors to their biological effects in granulosa cells. To test this hypothesis, we examined the effect of PRL and purified $(Ose)_n$Ins*P* moiety (from rat liver membranes) on granulosa cell 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (3β-HSD) enzyme activity. Results presented show that, in FSH-primed granulosa cells, PRL (40 nM) and $(Ose)_n$Ins*P* (5 µM) prevented gonadotropin-stimulated 3β-HSD activity. Furthermore, in undifferentiated granulosa cells where PRL receptors are absent, no effect of the hormone on 3β-HSD activity could be observed, whereas $(Ose)_n$Ins*P* (1–10 µM) inhibited enzyme activity in a dose-dependent manner.

Ovarian granulosa cell proliferation and differentiation appears to be initiated by the binding of the gonadotropin follicle stimulating hormone (FSH) to specific plasma membrane receptors, resulting in the elevation of intracellular cAMP and the subsequent activation of protein-kinase-A-mediated events implicated in the transformation of the immature cell to its fully mature counterpart (for a review see Hsueh et al., 1985; Amsterdam et al., 1989). This process is accompanied by the development of functional prolactin (PRL) receptors that are further increased thereafter by homologous up-regulation and by luteinizing hormone (Wang et al., 1979; Wang et al., 1980). In differentiated granulosa cells, PRL-receptor activation prevents gonadotropin-stimulated steroidogenesis (Dorrington and Gore-Langton, 1982; Gitay-Goren et al., 1989; Martel et al., 1990) and this effect is probably involved in the inhibition of ovarian follicular growth and anovulation observed during hyperprolactinemia (Mc Neilly, 1980).

A large body of evidence has been accumulated which indicate that PRL initiates its pleiotropic actions in granulosa and other cell types by binding to specific high-affinity receptors at the target-cell surface, but the cellular mechanism(s) by which PRL binding to these receptors initiates cellular responses are poorly understood (for a review see Kelly et al., 1991). Recently, a reduced number of observations have implicated diacylglycerol (DAG) and/or activation of protein kinase C (PKC) as intracellular mediators of PRL in liver (Buckley et al., 1986; Buckley et al., 1987; Buckley et al., 1988; Johnson et al., 1990; Crowe et al., 1991) and smooth-muscle cells (Sauro et al., 1989; Sauro and Zorn, 1991). Nevertheless, since PRL-receptor activation in these cells is not accompanied by inositol 1,4,5-trisphosphate generation and/or increases in intracellular calcium, it seems reasonable to conclude that the source of DAG generated in response to PRL may involve membrane phospholipids other than phosphatidylinositolbisphosphate (PtdInsP_2; Farese et al., 1988a; Chan et al., 1989; Exton, 1990). Evaluation of this hypothesis led us to investigate the possibility that PRL-receptor activation stimulates the cleavage by phosphodiest-

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Abbreviations. FSH, follicle-stimulating hormone; $(Ose)_n$PtdIns, oligosaccharide-phosphatidylinositol (glycosyl-phosphatidylinositol); $(Ose)_n$Ins*P*, phosphoinositol-oligosaccharide (inositol-phosphoglycan); PLC, phospholipase C; PRL, prolactin; PtdIns, phosphatidylinositol; PtdIns*P*, phosphatidylinositol 4-phosphate; PtdInsP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase.

Enzymes. 3β-Hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (EC 1.1.1.51).

erase of hormone-sensitive $(Ose)_n$PtdIns lipids in ovarian granulosa cells.

Hormone-receptor activated $(Ose)_n$PtdIns hydrolysis elicited by insulin (Saltiel and Cuatrecasas, 1986; Saltiel and Sorbara-Cazan, 1987; Mato et al., 1987a) and other regulatory ligands (Cozza et al., 1988; Farese et al., 1988b; Chan et al., 1989; Eardley and Koshland, 1991) results in the generation of myristoyl-DAG species (Saltiel and Sorbara-Cazan, 1987; Eardley and Koshland, 1991) and a soluble phosphoinositol-oligosaccharide (Ose_nIns*P*) moiety containing inositol, glucosamine, galactose and several phosphates (Saltiel et al., 1986; Mato et al., 1987a; Mato et al., 1987b; Gaulton et al., 1988). Although a second-messenger function for DAG species generated after $(Ose)_n$PtdIns hydrolysis has not been clearly established, in intact cells the inositol-glycan moiety is endowed with effects which mimic those of insulin in widespread intracellular events including inhibition of lipolysis (Kelly et al., 1987) stimulation of lipogenesis (Saltiel and Sorbara-Cazan, 1987) inhibition of cAMP-dependent protein phosphorylation (Larner et al., 1979), intracellular cAMP accumulation (Alvarez et al., 1987) and the phosphorylation/dephosphorylation of some of the proteins resembling insulin action (Larner et al., 1979; Alemany et al., 1987).

In the present investigation, we show that, in FSH-primed granulosa cells endowed with PRL receptors, the lactogenic hormone stimulates the rapid turnover of $(Ose)_n$PtdIns and generation of myristoyl-DAG. Furthermore, the soluble $(Ose)_n$Ins*P* moiety obtained by PtdIns-specific PLC treatment of $(Ose)_n$PtdIns extracted from rat liver membranes reproduces the inhibitory effects of PRL on FSH-stimulated granulosa cell 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (3β-HSD) and also inhibits enzyme activity in undifferentiated granulosa cells where PRL receptors are absent. This evidence suggest that PRL-receptor-activated phosphodiesterase cleavage of $(Ose)_n$PtdIns generates DAG and a soluble $(Ose)_n$Ins*P*-moiety that may be implicated in the inhibitory effects of PRL on granulosa cell function.

MATERIALS AND METHODS

Reagents and hormones

The pituitary hormones FSH (NIADDK-oFSH-16) and PRL (NIADDK-rPRL-B-5) were donated by the National Hormone and Pituitary Agency. The labelled compounds $NaB[^3H]_4$ (15 Ci/mmol), [6-^{3}H]galactose (46 Ci/mmol), [1-^{3}H]galactosamine (25 Ci/mmol), [6-^{3}H]glucosamine (30 Ci/mmol), [2-^{3}H]mannose (29.5 Ci/mmol), [2-^{3}H]myoinositol (14.6 Ci/mmol), [9,10-^{3}H]myristate (39.3 Ci/mmol), 1-arachidonyl, [2-^{3}H]stearoyl-*sn*-glycerol (60 Ci/mmol), [7-^{3}H]pregnenolone (20 Ci/mmol), [1-^{14}C]progesterone (51 Ci/mol), [1-^{14}C]myristic acid (50 Ci/mol) and human ^{125}I-PRL (50 μCi/μg) were purchased from Du Pont, New England Nuclear.

Phosphatidylinositol-specific phospholipase C (PLC) from *Bacillus cereus* was from Boehringer Mannheim and PLC from *Bacillus thuringiensis* was generously provided by Dr S. Udenfriend (Roche Institute of Molecular Biology, Nutley, USA). Galactose oxidase from *Doctylium dendroides*, β-galactosidase from *Escherichia coli*, phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns*P*), PtdInsP_2 and other lipid standards were from Sigma Chemical Co. The coated silica-gel plates were obtained from Merck and the high-performance thin-layer chromatography plates (HP-FK) were purchased from Whatman. Tissue-culture reagents and culture media were obtained from Gibco. The culture dishes (35 mm × 10 mm) and culture polystyrene tubes (12 mm × 75 mm) were purchased from Becton-Dickinson.

Cell culture and metabolic labelling

Granulosa cells were obtained by follicle puncture of the ovaries of immature (22–24-days old) diethylstilbestrol-implanted Sprague-Dawley rats (Fanjul et al., 1983; Fanjul et al., 1992). Briefly, the cells were collected into Mc Coy's 5a medium (modified without serum), transferred to sterile plastic tubes, washed twice by centrifugation, and the cell viability determined by trypan-blue exclusion (routinely, the dye was excluded from more than 80% of the cells).

Granulosa cells ($\approx 10^6$ viable cells/dish) were cultured for the time periods indicated for each experiment in 1 ml medium (supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine) in the presence of 10 μCi/ml ^{3}H-labelled carbohydrates (galactose, galactosamine, glucosamine, mannose and *myo*-inositol) or 5 μCi/ml [^{14}C]myristate. Hormones and other components, were added from the appropriate sterile stock solutions, freshly diluted in the same culture medium.

Phospholipid extraction and separation

To terminate the experiments, media were aspirated and 1 ml ice-cold 5% trichloroacetic acid was added to the cultures. After standing for 30 min at 4°C, the cells were scraped from the dishes, transferred to clean glass tubes and centrifuged (1000 × *g*) for 30 min at 4°C. The precipitate was extracted twice with 3 ml chloroform/methanol (1:2, by vol.) containing 0.03 M HCl, and the organic phases were pooled, dried under nitrogen, redissolved in chloroform/methanol (2:1, by vol.) and spotted on activated silica-gel plates. The plates were developed twice with the solvent system chloroform/acetone/methanol/glacial acetic acid/water (50:20:10:10:5, by vol.). In this acid-mobile phase, PtdInsP_2 remains at the origin and the other phospholipids migrate with R_F values of 0.12, 0.25 and 0.31 for PtdIns*P*, $(Ose)_n$PtdIns and PtdIns, respectively. The $(Ose)_n$PtdIns fraction was extracted from the silica with methanol at 37°C and rechromatographed in the mobile-phase chloroform/methanol/NH_4OH (45:45:3.5, by vol.). In this basic system, PtdIns*P* remains near the origin (R_F 0.05) and a good separation of $(Ose)_n$PtdIns and PtdIns was achieved (R_F 0.32 and 0.65, respectively).

In cells cultured in the presence of [^{3}H]*myo*-inositol, the phosphatidylinositols (PtdIns, PtdIns*P* and PtdInsP_2) were also labelled, and the [^{3}H]$(Ose)_n$PtdIns was further separated on high-performance TLC plates (Gaulton, 1991), with the mobile phase chloroform/methanol/NH_4OH/water (45:45:3.5:10, by vol.). In this solvent system, the [^{3}H]*myo*-inositol-labelled $(Ose)_n$PtdIns migrated as a single species. The dried plates were impregnated with EN^{3}HANCE (Du Pont, New England Nuclear) and exposed to Kodak X-AR film for 10 days at −70°C, the radioactivity associated with $(Ose)_n$PtdIns was then determined by liquid-scintillation counting.

Oligosaccharide-phosphatidylinositol characterization

Samples of the ^{3}H-labelled oligosaccharide-phosphatidylinositol purified from granulosa cells labelled with the dif-

ferent carbohydrates, were dried under nitrogen, resuspended in 0.2 ml 4 M HCl and the tubes sealed under vacuum. After a 5-h incubation at 110°C, 1 ml H_2O was added to each tube and the contents transferred to clean glass tubes. The extracts were lyophilised, redissolved in water, supplemented with the appropriate standards and chromatographed twice on activated silica-gel plates with the mobile phase pyridine/ethyl acetate/acetic acid/water (5:5:1:3, by vol.), then dried (Gaulton et al., 1987; Mato et al., 1987a; Gaulton, 1991). 1-cm regions were scraped into scintillation vials and the radioactivity associated with each portion determined by liquid-scintillation counting.

The sensitivity of granulosa cell (Ose)$_n$PtdIns to PLC hydrolysis and nitrous acid deamination was also investigated; the detailed procedures have been described elsewhere (Gaulton et al., 1988; Mato et al., 1987a; Mato et al., 1987b). Briefly, samples of [^{3}H](Ose)$_n$PtdIns were dried under nitrogen and incubated for 12 h at 37°C with 0.2 ml of 0.33 M sodium nitrite in 25 mM sodium acetate, pH 3.5, or the same volume of 20 mM sodium borate, pH 7.4, containing 1 U PtdIns-specific PLC from *B. cereus*. Results were corrected for non-specific conversion in control incubations conducted at pH 3.5 in the absence of sodium nitrite, or at pH 7.4 in the absence of PLC.

Labelling of granulosa cell and rat liver oligosaccharide-phosphatidylinositol with galactose oxidase and NaB[^{3}H]$_4$

The (Ose)$_n$PtdIns was purified from 10 rat livers or from granulosa cell membranes isolated from the ovaries of 50 rats by the sequential TLC procedure described, eluted from the plates, resuspended by sonication in 0.3 ml 50 mM sodium phosphate, pH 8.0, and treated with galactose oxidase (5 U) and 2 mCi NaB[^{3}H]$_4$ as described previously (Mato et al. 1987a; Alvarez et al. 1991). After a 1-h incubation at 37°C, the reaction mixture was chilled on ice, dried under vacuum, extracted with chloroform/methanol (2:1 by vol.) and the [^{3}H]galactose-labelled (Ose)$_n$PtdIns purified by sequential TLC with the acid and basic mobile phases described. The radiolabelled (Ose)$_n$PtdIns from granulosa cells or rat liver was extracted from the plates, dissolved in 0.75 ml chloroform/methanol/1 M HCl (2:1:0.03, by vol.) and 2 ml water added to form two phases (Alvarez et al., 1991). After vigorous shaking, the organic phase was removed, the water phase washed twice with 0.3 ml chloroform/methanol (2:1 by vol.) and the organic phases combined and dried under vacuum. TLC analysis of the [^{3}H]galactose-labelled (Ose)$_n$PtdIns from granulosa cells or rat liver comigrated with the same R_F as [^{3}H](Ose)$_n$PtdIns extracted from cell cultures which had ben metabolically labelled with galactose, glucosamine and inositol (results not shown).

The (Ose)$_n$PtdIns was further purified by high-performance liquid chromatography using a silica column (Ultrasil-Si, 10-µm pore size, 4.6 mm×250 mm; Beckman Instruments). The (Ose)$_n$PtdIns was eluted with a 20-min linear gradient from 100% A (chloroform/methanol/glacial acetic acid; 14:2:1, by vol.) to 100% B (chloroform/methanol/glacial acetic acid/water; 40:45:10:2, by vol.) at a flow rate of 1 ml/min, as described previously (Alvarez et al., 1991), and the radioactivity associated with each 0.4-ml fraction determined by liquid-scintillation counting (Fig. 1). The labelled (Ose)$_n$PtdIns was evaporated to dryness under nitrogen, resuspended in 0.2 ml 50 mM Tris/HCl, pH 7.4, containing 1 U β-galactosidase, and incubated for 6 h at 37°C. Reactions were terminated by adding 0.75 ml chloroform/methanol/1 M HCl (2:1:0.03, by vol.), followed by 2 ml water. After vigourous shaking, the amount of radioactivity released into the aqueous phase was determined by liquid-scintillation counting. In parallel experiments, [^{3}H]galactose-labelled (Ose)$_n$PtdIns from granulosa cells or rat liver membranes were further characterized by PtdIns-specific PLC hydrolysis and nitrous acid deamination (Table 1).

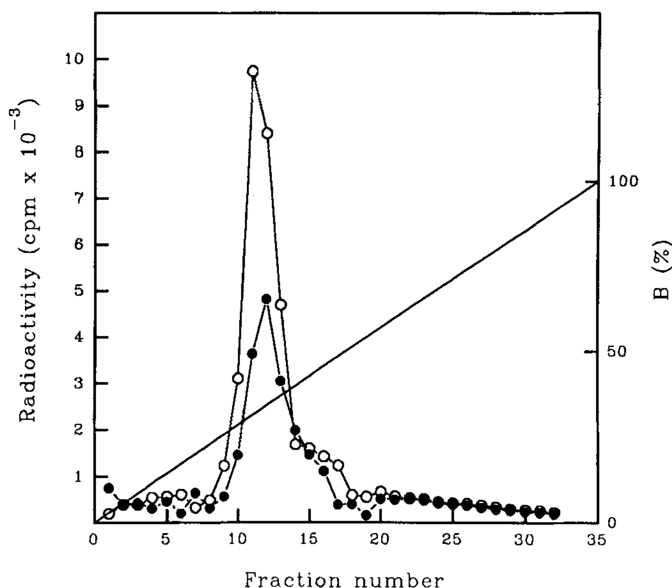

Fig. 1. Analysis of granulosa cell and rat liver [^{3}H](Ose)$_n$PtdIns by high-performance liquid chromatography. The (Ose)$_n$PtdIns extracted from granulosa cells or rat liver was labelled with galactose oxidase and NaB[^{3}H]$_4$ as described in Materials and Methods and analyzed by HPLC using a silica-gel column (Ultrasil-Si, 10-µm pore size, 4.6 mm×250 mm; Beckman Instruments). Aliquots of [^{3}H]galactose-labelled (Ose)$_n$PtdIns from granulosa cells (○) or rat liver (●) were eluted with a 20-min linear gradient from 100% A (chloroform/methanol/glacial acetic acid; 14:2:1, by vol.) to 100% B (chloroform/methanol/glacial acetic acid/water; 40:45:10:2, by vol.). The radiolabelled (Ose)$_n$PtdIns was eluted at a flow rate of 1 ml/min and the radioactivity associated in each 0.4-ml fraction determined by liquid-scintillation counting.

Purification of the phosphoinositol-oligosaccharide moiety from rat liver

Since preliminary experiments revealed that granulosa cell (Ose)$_n$PtdIns is structurally related to that reported in other cell types (Saltiel and Cuatrecasas, 1986; Mato et al., 1987a; Mato et al., 1987b; Eardley and Koshland, 1991), rat liver membranes were used as an abundant source of (Ose)$_n$-InsP. The (Ose)$_n$PtdIns was isolated by the sequential TLC procedure described, eluted from the plates, resuspended by sonication in 0.2 ml sodium borate buffer and treated with 1 U (defined as the amount of enzyme that hydrolyses 0.8 nmol PtdIns in 1 min at 37°C) PLC from *B. thuringiensis* for 12 h at 37°C (Mato et al., 1987a; Villalba et al., 1990; Alvarez et al., 1991). The reaction was stopped with 0.75 ml chloroform/methanol/1 M HCl (2:1:0.03, by vol.) and the polar (Ose)$_n$Ins*P* moiety isolated from the aqueous phase as described elsewhere (Mato et al., 1987a; Alvarez et al., 1991). The concentration of (Ose)$_n$Ins*P* was calculated as previously described (Varela et al., 1990) and the biological activity determined by testing its capacity to inhibit the phosphorylation of histone IIA by the cAMP-dependent protein

Table 1. Characterization of [^{3}H]galactose-labelled (Ose)$_n$PtdIns from granulosa cell and rat liver membranes. (Ose)$_n$PtdIns extracted from rat liver and granulosa cells, were treated with galactose oxidase (5 U) and 2 mCi of NaB[^{3}H]$_4$ as described in Materials and Methods, and the [^{3}H]galactose-labelled (Ose)$_n$PtdIns further purified by high-performance TLC and high-performance liquid chromatography (Fig. 4). Equivalent amounts (5000−10000 cpm) of the purified [^{3}H](Ose)$_n$PtdIns from granulosa cells or rat liver, were dried under a stream of nitrogen and resuspended in 0.2 ml 50 mM Tris/HCl, pH 7.4, containing 1 U β-galactosidase. After 6 h incubation at 37°C, reactions were terminated by adding 0.75 ml chloroform/methanol/1 M HCl (2:1:0.03, by vol.), followed by 2 ml water. After vigorous shaking, the amount of radioactivity released into the aqueous phase was determined by liquid-scintillation counting. Mock incubations were performed in buffer alone and results corrected for non-specific conversion of radioactivity. In parallel experiments, galactose oxidase and NaB[^{3}H]$_4$-labelled (Ose)$_n$PtdIns from granulosa cell or rat liver membranes were further characterized by PtdIns-specific PLC hydrolysis and nitrous acid deamination, exactly as described in Table 2. Results were expressed as a percentage of the radioactivity recovered in the aqueous phase, and similar results were obtained in three other experiments.

[^{3}H](Ose)$_n$PtdIns	Radioactivity released from (Ose)$_n$PtdIns by		
	β-galactosidase	nitrous acid	PtdIns-PLC
	%		
Granulosa cell	96	88	77
Rat liver	93	91	84

Table 2. Characterization of granulosa cell oligosaccharide-phosphatidylinositol. Granulosa cells were cultured for 48 h with 10 µCi/ml [^{3}H]glucosamine, [^{3}H]galactose or [^{3}H]inositol and the labelled (Ose)$_n$PtdIns was extracted and purified as described in Materials and Methods. Samples (3000−5000 cpm) of [^{3}H](Ose)$_n$PtdIns were subjected to acid hydrolysis and the aqueous extracts and the appropriate standards were applied to TLC plates and developed twice in the system pyridine/ethyl acetate/acetic acid/water (5:5:1:3, by vol.). The results are expressed as a percentage of radioactivity that comigrated with the same retardation factor as inositol (R_F 0.26), glucosamine (R_F 0.50) and galactose (R_F 0.88). Parallel samples of [^{3}H](Ose)$_n$PtdIns, were incubated with sodium nitrite, pH 3.5, or PtdIns-specific PLC. Results, expressed as a percentage of the radioactivity released into the aqueous phase, were corrected for non-specific conversion determined in control incubations conducted at pH 3.5 in the absence of sodium nitrite, or at pH 7.4 in the absence of PtdIns-PLC from *B. cereus*. After incubation of [^{3}H]PtdIns with the same PLC, 75−85% of the radioactivity was released into the aqueous phase. Similar results were obtained in three other experiments.

[^{3}H]Saccharide	Radioactivity released from (Ose)$_n$PtdIns by		
	acid hydrolysis	nitrous acid	PtdIns-PLC
	%		
Glucosamine	92	62	62
Galactose	85	75	72
Inositol	93	6	80

kinase (Villalba et al., 1988). Samples of purified (Ose)$_n$Ins*P* were also treated with nitrous acid (Villalba et al., 1988) and used in control incubations.

Diacylglycerol determination

Granulosa cells were cultured for 3 days in the presence of FSH (30 ng/ml) and labelled for the last 5 hours with [^{14}C]myristate (5 µCi/ml). The media were replaced with warm (37°C) fresh media and the cultures allowed to equilibrate for an additional 30-min period. After this period, PRL (40 nM) or FSH (30 ng/ml) were added from appropriate stock solutions in a retrograde fashion to triplicate or quadruplicate cultures. The cells were scraped into methanol supplemented with [^{3}H]DAG (≈1500 dpm) to correct for losses and separated by the TLC procedure described (Fanjul et al., 1992).

3β-HSD assay

In selected experiments, media were aspirated and granulosa cells scraped from the dishes into cold assay buffer (0.05 M potassium phosphate, 1 mM EDTA, pH 7.4) sonicated at 4°C for 15 s at 50% setting in an ultrasonicator (Labsonic 2000) and aliquots of cell homogenates from individual dishes (20−80 µg protein) assayed for 3β-HSD activity as the rate of conversion of [^{3}H]pregnenolone (50 µM, ≈10^4 cpm/nmol) to [^{3}H]progesterone in the presence of a saturating concentration (1 mM) of NAD$^+$ (Ruiz de Galarreta et al., 1983; Fanjul et al., 1992). Protein concentrations were determined using the Bio-Rad dye-binding assay, with bovine γ-globulin as standard.

PRL-receptor assay

The binding assay was performed for 16 h at room temperature (Wang et al., 1979; Wang et al., 1982) with a saturating amount of ^{125}I-PRL (≈ 100000 cpm) with or without of an excess amount of unlabelled PRL (1 µg/tube). Non-specific binding (three determinations/ treatment condition) was subtracted from the total binding (four determinations) to derive the amount of specifically bound hormone.

Statistical analysis

Results are expressed as mean ± SEM from triplicate or quadruplicate cultures and experiments replicated at least three times. Statistical analysis were performed using analysis of variance (ANOVA) and, as indicated, the Student's *t*-test for comparison of the means. Comparisons with $P > 0.05$ were not considered significant.

RESULTS AND DISCUSSION

The possibility that PRL-receptor activation stimulates the cleavage by phosphodiesterase of membrane (Ose)$_n$PtdIns was investigated. Granulosa cells were labelled with putative precursors and (Ose)$_n$PtdIns isolated by chromatography in the acid and basic mobile phases described (Gaulton et al., 1988; Mato et al., 1988a, 1988b) and further characterized by acid hydrolysis, bacterial PLC treatment and nitrous acid deamination (Table 2). The isotopic integrity after metabolic labelling was evaluated by TLC analysis of an acid hydrolysate of the putative [^{3}H](Ose)$_n$PtdIns (Mato et al., 1987a; Gaulton et al., 1988). No radioactivity was detected in (Ose)$_n$PtdIns when granulosa cells were incubated with [^{3}H]galactosamine, [^{3}H]mannose or [^{3}H]sorbitol (results not

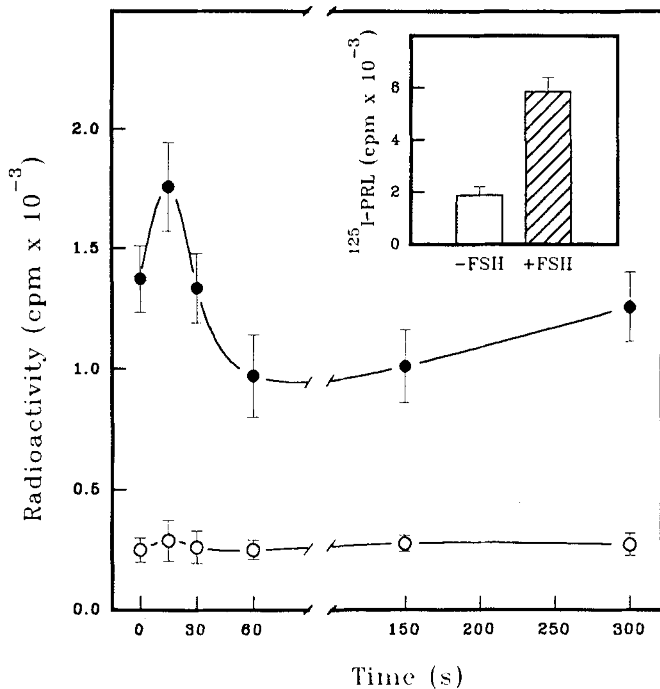

Fig. 2. Effects of PRL on (Ose)$_n$PtdIns turnover in granulosa cells. Granulosa cells (≈ 10^6 viable cells/dish) were labelled with [^{3}H]glucosamine (10 μCi/ml) for 72 hours, in the absence (○) or presence (●) of 30 ng/ml FSH to induce differentiation. After 3 days of culture, PRL (final concentration 40 nM) was added to triplicate or quadruplicate cultures in a retrograde fashion and the radioactivity associated with (Ose)$_n$PtdIns, isolated from each culture, determined by liquid-scintillation counting. In parallel experiments, cells were cultured in polystyrene tubes and specific ^{125}I-PRL binding determined as described in Materials and Methods. Results are mean ± SEM of triplicate or quadruplicate cultures from three different experiments. No increase in [^{3}H](Ose)$_n$PtdIns labelling was observed in cells cultured for 72 h in the presence of 40 nM PRL or 100 ng/ml human chorionic gonadotropin (results not shown).

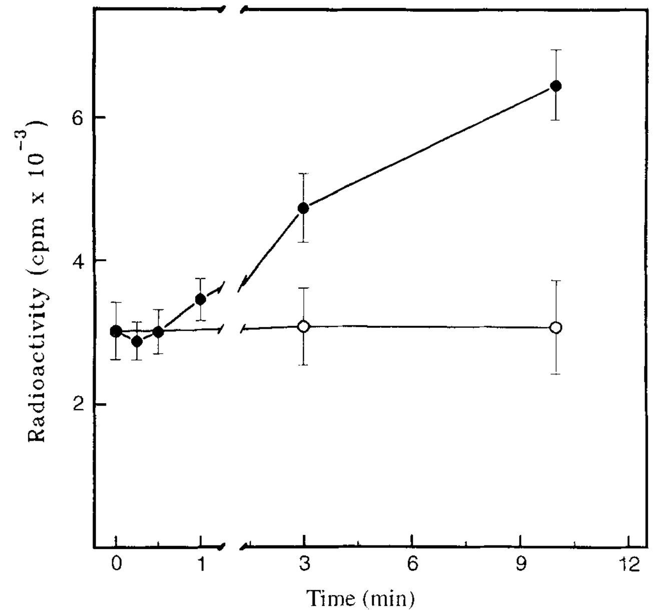

Fig. 3. Prolactin stimulation of granulosa cell DAG generation. Granulosa cells (≈ 10^6 viable cells/dish), were cultured for 72 h with FSH (30 ng/ml) and labelled with [^{14}C]myristate (5 μCi/ml) for the last 5 h. The cultures were washed, allowed to equilibrate for 30 min and FSH (○) or PRL (●), freshly diluted in the same amount of culture medium (0.1 ml), were added to each culture. The cells were scraped into methanol supplemented with [^{3}H]DAG (≈ 1500 dpm) to correct for losses and DAG was separated by the TLC procedure described in Materials and Methods. Results are the mean ± SEM of quadruplicate dishes. Similar results were obtained in three additional experiments.

shown). After treatment of [^{3}H]glucosamine-, [^{3}H]galactose- or [^{3}H]myoinositol-labelled (Ose)$_n$PtdIns with PtdIns-specific PLC from *B. cereus*, 60–80% of the radioactivity was released into the aqueous phase. Approximately 75% of the radioactivity was recovered in the aqueous phase after nitrous acid deamination of (Ose)$_n$PtdIns labelled with [^{3}H]glucosamine or [^{3}H]galactose, whereas radioactivity released after $NaNO_2$ treatment of [^{3}H]*myo*-inositol-labelled (Ose)$_n$PtdIns or [^{3}H]PtdIns used as control was negligible (Table 2). The structural similarity of granulosa cell and rat liver (Ose)$_n$PtdIns was also investigated. The terminal galactose of (Ose)$_n$PtdIns isolated from granulosa cells or rat liver was labelled with galactose oxidase and NaB[^{3}H]$_4$ (Alvarez et al., 1991) and purified by sequential TLC followed by HPLC. Approximately 80–90% of the original radioactivity of the (Ose)$_n$PtdIns eluted as a single peak from the HPLC column, with a retention time of about 8 min (Fig. 1). The [^{3}H]galactose-labelled (Ose)$_n$PtdIns from granulosa cells or rat liver, showed similar sensitivities to β-galactosidase and PtdIns-specific PLC hydrolysis, or nitrous acid deamination (Table 1), and comigrated with the same retention factor in the basic TLC system as (Ose)$_n$PtdIns extracted from cells metabolically labelled with galactose, glucosamine or inositol (results not shown).

The level of target-cell PRL-receptor expression is subjected to hormonal regulation in granulosa cells (Hsueh et al., 1985), and other cell types (Kelly et al., 1991). As shown in Fig. 2, treatment with FSH stimulated the acquisition of PRL receptors and increased the (Ose)$_n$PtdIns content in granulosa cells. Similar increases in (Ose)$_n$PtdIns content have also been reported after phytohaemmagglutinin-induced increase in insulin-receptor number in lymphocytes (Gaulton et al., 1988) or in chinese hamster ovary cells overexpressing the human insulin receptor (Villalba et al., 1990). Since no effect on (Ose)$_n$PtdIns content could be observed when granulosa cells were labelled for 3 days in the presence of PRL or human chorionic gonadotropin (results not shown), it is tempting to speculate that the observed effect of FSH on the (Ose)$_n$PtdIns content appears to be a common feature of receptor up-regulation associated with hormones that activate cleavage of (Ose)$_n$PtdIns by phosphodiesterase.

In cultured FSH-primed granulosa cells endowed with receptors for lactogen, treatment with PRL stimulated the rapid hydrolysis of the lipid (Fig. 2) and the generation of myristoyl-DAG (Fig. 3), whereas no effect of the hormone could be observed in undifferentiated cells.

The rapid and transient hydrolysis of (Ose)$_n$PtdIns (Fig. 2) contrasted with the prolonged elevation of DAG observed after prolactin treatment of granulosa cells (Fig. 3) and raises the interesting possibility that PRL-induced DAG generation implicates cleavage of (Ose)$_n$PtdIns as well as additional membrane lipids by phosphodiesterase (Exton, 1990). Nevertheless, the possibility that the long lasting effect of prolactin on DAG generation may also involve phosphoinositide turnover can be ruled out, since in [^{3}H]*myo*-inositol-labelled granulosa cells PRL-stimulated hydrolysis of

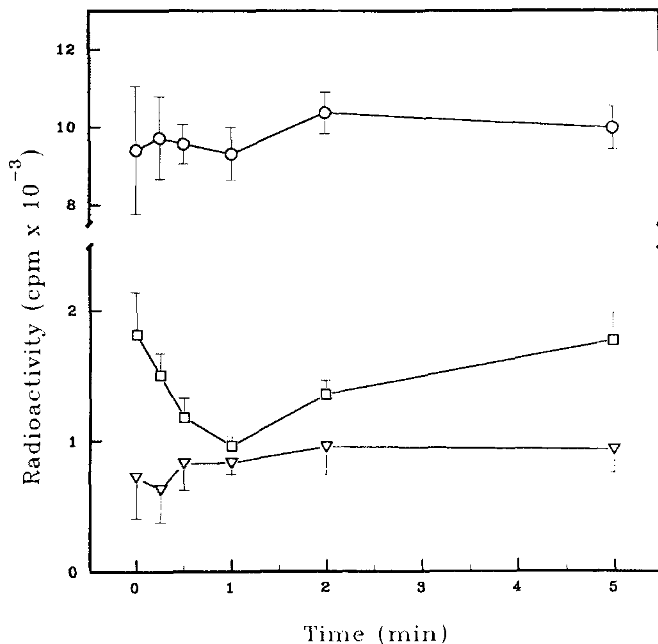

Fig. 4. Effects of prolactin on granulosa cell $(Ose)_n$PtdIns and phosphoinositide (PtIns, PtIns*P* and PtInsP_2) turnover. Granulosa cells ($\approx 10^6$ viable cells/dish) were labelled with [^{3}H]*myo*-inositol (10 µCi/ml) for 3 days in the presence of FSH (30 ng/ml). After this period, the culture media was replaced, cells allowed to equilibrate and PRL (final concentration 40 nM) added in a retrograde fashion to triplicate or quadruplicate cultures. The lipids were extracted, separated by TLC as described in Materials and Methods and the radioactivity associated with PtIns (○), PtInsP_2+PtIns*P* (△) and $(Ose)_n$PtdIns (□), determined by liquid-scintillation counting. Results represent mean ± SEM from triplicate cultures of an experiment representative from three other experiments.

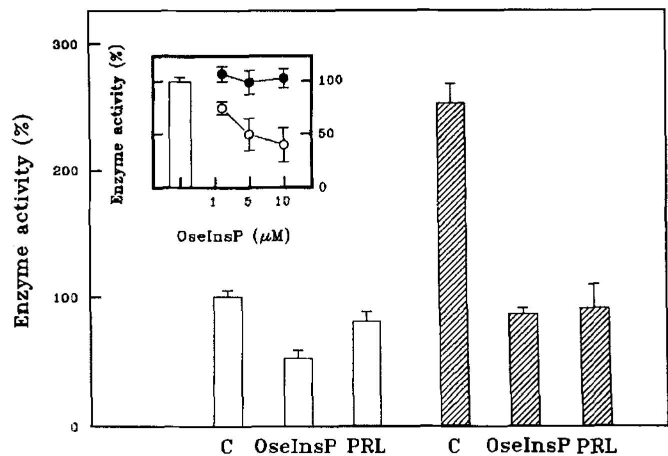

Fig. 5. Effects of PRL and $(Ose)_n$Ins*P* on granulosa cell 3β-HSD activity. Granulosa cells ($\approx 5 \times 10^5$ viable cells/dish) were cultured for 48 h in the absence (open bars) or presence (dashed bars) of FSH (30 ng/ml) in culture medium supplemented with PRL (40 nM) or $(Ose)_n$Ins*P* (5 µM) obtained by enzymic hydrolysis of rat liver $(Ose)_n$PtdIns as described in Materials and Methods. After this period, media were aspirated, cells scraped from the dishes and the enzyme activity determined in cell homogenates. Control incubations with the same concentration of $(Ose)_n$Ins*P* in the enzyme assay did not affect [^{3}H]progesterone formation (results not shown). Granulosa cells were also cultured for 48 h in the presence of increasing concentrations (1–10 µM) $(Ose)_n$Ins*P* (○) or equivalent amounts of the $(Ose)_n$Ins*P* subjected to nitrous acid deamination (●) and enzyme activity determined in the same manner (inset panel). Results represent mean ± SEM of quadruplicate cultures from an experiment representative of three others.

[^{3}H]$(Ose)_n$PtdIns was not accompanied by phosphoinositide (PtdIns, PtdIns*P* and PtdInsP_2) turnover (Fig. 4). A similar sustained effect on DAG generation has been reported after gonadotropin-releasing hormone stimulated PtdInsP_2 hydrolysis in granulosa cells, and has been related to the sequential stimulation of other guanosine-nucleotide-binding-protein-coupled phospholipases present in the granulosa cell (Liscovitch and Amsterdam, 1989; Fanjul et al., 1992). Identification of these additional lipids may aid our understanding of the cellular mechanism(s) mediating granulosa cell differentiation; this would be the subject of an entirely separate study.

In the same experiments, the rapid (60 s) decrease ($\approx$ 50%) in $(Ose)_n$PtdIns-associated radioactivity detected in prolactin-treated [^{3}H]*myo*-inositol-labelled granulosa cells (Fig. 4), contrasted with the small initial increase in the $(Ose)_n$PtdIns-associated radioactivity observed in previous experiments (Fig. 2), probably reflecting the inability of the cells to achieve isotopic equilibrium after metabolic labelling with [^{3}H]glucosamine.

Treatment with increasing concentrations of PRL (10–80 nM) had no effect on progesterone production and 3β-HSD activity of undifferentiated cells where PRL receptors are absent (results not shown), and a 40-nM concentration of the hormone maximally prevented FSH-stimulated 3β-HSD enzyme activity of granulosa cells (Fig. 5). A similar undetectable influence of PRL on aromatase and 3β-HSD enzyme activity and gene expression in unstimulated granulosa cells, contrasted with the inhibitory effect of the hormone on gonadotropin-stimulated steroidogenesis reported in the same experiments (Dorrington and Gore-Langton, 1982; Gitay-Goren et al., 1989; Krasnow et al., 1990; Martel et al., 1990).

The differentiation of the granulosa cell begins with the binding of FSH to specific plasma membrane receptors with subsequent stimulation of adenylate cyclase and augmentation of cytoplasmic cAMP-dependent phosphorylation of regulatory proteins (Knecht et al., 1981; Knecht et al., 1983; Ben-Ze'ev and Amsterdam, 1987). Depending on the stage of maturation of the granulosa cell after induction with gonadotropin, receptor-activated DAG generation or the direct activation of PKC with phorbol esters, inhibits FSH-induced granulosa cell function (Shinahora et al., 1985; Ben-Ze'ev and Amsterdam, 1987; Fanjul et al., 1992) or stimulates steroidogenesis in the undifferentiated cell (Shinohara et al., 1985; Amsterdam et al., 1989; Fanjul et al., 1992). Results presented here suggest that generation of DAG and a soluble $(Ose)_n$Ins*P* moiety could be part of the signal-transduction mechanism linking the PRL receptor with its biological effects, as has been proposed to explain the paradoxical role of the DAG/PKC pathway mediating insulin and nerve growth factor action in the absence of phosphoinositide turnover (Saltiel and Decker, 1991).

Since the $(Ose)_n$Ins*P* moiety has widespread metabolic effects in different cell types (for a review see Kilgour, 1993), we next tested the effect of $(Ose)_n$Ins*P* on granulosa cell function. Granulosa cell $(Ose)_n$PtdIns meets the basic structural requirements of $(Ose)_n$PtdIns isolated from other cell types and, therefore, the structurally related $(Ose)_n$PtdIns from rat liver membranes (Fig. 1 and Table 1) was used as an abundant source of $(Ose)_n$Ins*P*.

Table 3. Effect of concurrent treatment with PRL and inositol-phosphoglycan on 3β-HSD activity of untreated and gonadotropin-primed granulosa cells. Granulosa cells ($\approx 5 \times 10^5$ viable cells/dish) were cultured for 48 h with and without FSH (30 ng/ml) in the absence or presence of different concentrations of PRL (40 nM and 80 nM), $(Ose)_n InsP$ (5 µM and 10 µM) or combinations thereof. After this period, media were aspirated and cells scraped from the dishes and assayed for enzyme activity in cell homogenates using described methods. Similar results for enzyme activity (mean ± SEM) were obtained in two other experiments.

Treatment	3β-HSD activity in	
	control cells	FSH-primed cells
	nmol progesterone formed $\cdot$ 30 min^{-1} $\cdot$ mg protein^{-1}	
None	4.43 ± 0.16	12.23 ± 0.96
PRL (40 nM)	4.19 ± 0.13	4.23 ± 0.56
PRL (80 nM)	4.62 ± 0.31	4.63 ± 0.86
$(Ose)_n InsP$ (5 µM)	2.24 ± 0.11	4.44 ± 0.33
$(Ose)_n InsP$ (10 µM)	2.19 ± 0.09	3.99 ± 0.44
PRL (40 nM) + $(Ose)_n InsP$ (5 µM)	2.27 ± 0.06	4.68 ± 0.56
PRL (40 nM) + $(Ose)_n InsP$ (10 µM)	2.30 ± 0.02	4.22 ± 0.14
PRL (80 nM) + $(Ose)_n InsP$ (5 µM)	1.95 ± 0.11	4.32 ± 0.27
PRL (80 nM) + $(Ose)_n InsP$ (10 µM)	2.25 ± 0.04	4.25 ± 0.16

A maximal (75%) inhibitory effect on 3β-HSD enzyme activity was observed in FSH-treated granulosa cells cultured in the presence of 5 µM $(Ose)_n InsP$ or 40 nM prolactin (Fig. 5), and no further effect on enzyme activity was observed when granulosa cells were cultured in the presence of maximal inhibitory doses of $(Ose)_n InsP$ and PRL (Table 3). Interestingly, in undifferentiated cells where lactogenic receptors are absent, increasing concentrations of $(Ose)_n InsP$ (1−10 µM) inhibited 3β-HSD activity in a dose-dependent manner. The specificity of $(Ose)_n InsP$ was also tested; no effect on enzyme activity could be observed in cells treated with equivalent amounts of $(Ose)_n InsP$ subjected to nitrous acid deamination (Fig. 5). The polar moiety of the lipid also did not interfere with the enzyme assay (results not shown).

These results contrast with a recent report showing that, in another steroidogenic cell model, the human placental cytotrophoblast cells, insulin mediators (purified from bovine liver), stimulated the conversion of exogenous pregnenolone to progesterone and inhibited aromatase activity in a manner similar to that of insulin (Nestler et al., 1991). The reason for these discrepancies could be due to differences in the source of $(Ose)_n InsP$ used and/or may simply reflect cell specificity. In this regard, it is worthwhile noting that insulin does not directly regulate steroid biosynthesis in rat granulosa cells, where insulin receptors are absent (for a review see Adashi et al., 1985). An alternative, but not necessarily mutually exclusive explanation for these differences, could be attributed to the experimental approach used to evaluate the steroidogenic activity of the cells. In the present investigation, we measured progesterone production in cells cultured under pregnenolone-free conditions (results not shown) and the 3β-HSD activity directly in cell homogenates (Fig. 5 and Table 3). In contrast, 3β-HSD activity in placental cytotrophoblasts was evaluated as the rate of progesterone produced by cells cultured in the presence of saturating amounts of exogenously added pregnenolone (Nestler et al., 1991).

The relative roles of DAG and $(Ose)_n InsP$, generated after PRL-stimulated $(Ose)_n InsP$ hydrolysis, are difficult to assess. Nevertheless, it is important to note that, in undifferentiated granulosa cells treated with PLC, generation of DAG activates 3β-HSD activity (Fanjul et al., 1992), whereas $(Ose)_n$-$InsP$ inhibits 3β-HSD activity in undifferentiated cells in a dose-dependent manner (Fig. 5). Furthermore, in FSH-primed granulosa cells, PRL inhibition of 3β-HSD activity was unaffected by the presence of $(Ose)_n InsP$ (Table 3). The present results suggest that the rapid PRL-stimulated generation of the $(Ose)_n InsP$ moiety could be implicated in the early modulation of the cAMP-PKA pathway (Larner et al., 1979; Saltiel and Cuatrecasas, 1986; Saltiel et al., 1986; Alvarez et al., 1987; Saltiel, 1987; Villalba et al., 1988), whereas the prolonged generation of DAG could be responsible for the long-term inhibitory effects of the hormone on granulosa cell function. This hypothesis seems reasonable since (a) the $(Ose)_n InsP$ moiety has been shown to regulate widespread intracellular events (Kelly et al., 1987; Saltiel and Sorbara-Cazan, 1987; Alvarez et al., 1987; Alemany et al., 1987) including stimulation of cAMP phosphodiesterase (Saltiel and Cuatrecasas, 1986; Saltiel et al., 1986) as well as inhibition of adenylate cyclase and cAMP-dependent protein kinase activity (Alvarez et al., 1987; Saltiel, 1987; Villalba et al., 1988); (b) the inhibitory effect of PRL on gonadotropin-stimulated steroidogenesis and cAMP accumulation is abolished in granulosa cells by the phosphodiesterase inhibitor MIX (Dorrington and Gore-Langton, 1982; Gitay-Goren et al., 1989; Martel et al., 1990) and (c) the generation of DAG represents a mechanism whereby Ca^{2+}-independent PKC isoforms can regulate gonadotropin-induced maturation and steroidogenic function of the granulosa cell in the absence of $PtInsP_2$ hydrolysis or Ca^{2+} mobilization (Fanjul et al., 1992).

Receptor-activated $(Ose)_n PtdIns$ hydrolysis has been associated with hormones or growth factors that bind to receptors with tyrosine kinase activity (Chan et al., 1989; Saltiel, 1987; Farese et al., 1988a; Farese et al., 1988b; Gaulton et al., 1988; Eardley and Koshland, 1991) or receptors with seven transmembrane domains coupled to cAMP generation (Cozza et al., 1988; Martiny et al., 1992). The lactogenic-receptor family has recently been included in the haematopoietin-cytokine-receptor superfamily involved in the growth and differentiation of cells of the lymphohaematopoietic lineages (Bazan, 1989; Bazan, 1990). Cloning and expression studies revealed that the haematopoietic receptor superfamily is distinct from guanosine-nucleotide-binding-protein associated hormone receptors or receptors expressing a tyrosine kinase in their cytoplasmic domains (Bazan, 1989; Bazan, 1990; Kelly et al., 1991; Waldmann, 1991). It is worthwhile noting that the interleukin-2 receptor activation in B lymphocytes stimulates receptor-unit association and interaction with specific $p56^{lck}$ protein tyrosine kinase, leading to the phosphorylation of the receptor β chain (Waldmann, 1991) and the rapid cleavage of a $(Ose)_n PtdIns$ of similar composition to granulosa cell $(Ose)_n PtdIns$, followed by proliferation of lymphoma cells (Too et al., 1989; Eardley and Koshland, 1990; Mérida and Gaulton, 1990; Waldmann, 1991). Since growth hormone (Carter-Su et al., 1989) and PRL (Rillema et al., 1992) -receptor activation stimulates tyrosine kinase activity in target cells (Foster et al., 1988; Stred et al., 1992), it is tempting to speculate that a similar mecha-

nism could be involved in PRL-stimulated $(Ose)_n$PtdIns hydrolysis.

Definitive evidence for the transducing function of the $(Ose)_n$PtdIns system in PRL action will require the demonstration that activation of lactogenic receptors in other target cells also stimulates $(Ose)_n$PtdIns hydrolysis and/or that the soluble $(Ose)_n$Ins*P* moiety can stimulate the activity of enzymes identified to have PRL action, or stimulate the same growth-related genes reported for the hormone (Kelly et al., 1991). Nevertheless, the finding that another member of the cytokine-receptor superfamily stimulates cleavage of $(Ose)_n$-PtdIns by phosphodiesterase in granulosa cells is important by itself and, to our knowledge, is the first report demonstrating an intracellular messenger for PRL action at the target-cell level.

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