Inhibition by Prolactin of Membrane-Associated Phosphatidylinositol Kinase of Human Endometrial Fibroblast (43689)

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Abstract. **Certain malignant tumors synthesize and secrete a putative peptide mitogen, which elicits a potent proliferative response in their supporting stromal cells. We recently demonstrated that prolactin (PRL) binds to human endometrial fibroblasts and inhibits mitogenicity of an endometrial carcinoma extract (Imai A, *et al.* Proc Soc Exp Biol Med 203:117–122, 1993). In this report, we have studied inhibitory regulation by PRL of phosphatidylinositol (PtdIns) kinase activity associated with plasma membranes isolated from human endometrial fibroblasts. Incubation of the isolated plasma membrane with [γ-^{32}P]ATP and exogenous PtdIns caused [^{32}P]phosphate incorporation into PtdIns phosphate (PtdIns*P*); 95% of the ^{32}P-labeled PtnIns*P* was accounted for by PtdIns 4-*P*. The PtdIns phosphorylation by membrane preparations was selectively stimulated in a dose-dependent manner by vanadate, in parallel with an elevated autophosphorylation of endogenous membrane proteins. Concomitant exposure of the membrane preparations to PRL led to a remarkable inhibition of the vanadate-responsive PtdIns phosphorylation and protein autophosphorylation. This inhibition was dependent on PRL dose, and half-maximal effect occurred at a concentration 1–10 n*M* of PRL. Degradation of the produced PtdIns*P* in the plasma membranes was not affected by PRL. Similar inhibition of PtdIns kinase activities were observed in membranes prepared from cells that had been pretreated *in vivo* with PRL prior to assay *in vitro*. These findings demonstrate that PtdIns kinase activity associated with protein autophosphorylation is suppressed by PRL in plasma membrane isolated from endometrial fibroblasts. The inhibition of vanadate-responsive PtdIns kinase by PRL suggests an involvement of this enzyme in the antimitogenic action of the hormone on human endometrial fibroblasts.** [P.S.E.B.M. 1994, Vol 205]

Polyphosphoinositides constitute less than 0.1% of total cellular lipids, yet accumulating evidence suggests that the phosphoinositide has functions in the cellular growth and proliferation in addition to their role as the second messenger precursor (1–3). Phosphatidylinositol (PtdIns) kinases catalyze an early step in the polyphosphoinositide synthesis by the step-wise phosphorylation from PtdIns. Binding of certain hormones and growth factors to their specific receptors results in the increase in both polyphosphoinositides containing phosphate at the 3-position of the inositol ring and "traditional" 4-phosphorylated phosphoinositides (2–5). Several tyrosine kinase-containing growth factor receptors have been demonstrated to physically also associate with and activate the PtdIns kinase introducing phosphate into the 3-position (2, 3, 6–11). The PtdIns 3- and 4-kinase activities might, thereby, be important components of the mitogenic response.

We recently demonstrated that prolactin (PRL) blocks the mitogenic activity of the fibroblasts stimulated by the endometrial carcinoma-derived mitogen

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via a PRL receptor-mediated mechanism (12). As a first step in the direction of antigrowth activity of PRL, in the present study, we addressed the question of a possible interaction between PRL receptor and PtdIns kinase activity in the human endometrial fibroblasts. Our data reveal that in the plasma membrane PRL suppressed the PtdIns kinase activity stimulated by vanadate, known to stimulate DNA synthesis and the tyrosine kinase-coupled PtdIns kinase (13, 14).

Materials and Methods

Human Endometrial Fibroblast Preparation. Specimens of normal endometrium were obtained from patients undergoing elective hysterectomy for uterine liomyoma or dilatation and curettage as a screening test for carcinoma (12). The tissues were trimmed and washed with Hanks' Balanced Salt Solution (HBSS), and subsequently treated for 1.5 hr at 37°C with 0.25% collagenase (Type I) in HBSS. The cell suspension was filtered through nylon mesh to remove tissue fragments, cell debris, and fibers. The filtrate was then centrifuged and washed with HBSS. The cells in the pellet were resuspended and incubated in modified minimum essential medium, Eagles (MEM) supplemented with 10% fetal bovine serum (FBS). After several days of incubation, nonadherent cells were removed, and the fibroblasts were grown as a monolayer. No significant difference was detected between fibroblasts from secretory phase-endometrium and in the cells from proliferative phase-endometrium in our assay.

In some experiments, at confluence, monolayer cultures were preincubated in MEM with 1% FBS for two days and submitted to concurrent exposure to 100 n*M* PRL for 10 min.

Plasma Membrane Isolation. The procedure described before (15) was used to obtain purified plasma membrane fraction from the endometrial fibroblasts. The isolated plasma membranes were finally resuspended in lysis buffer (1 m*M* ethyleneglycol tetraacetic acid (EGTA), 1 m*M* dithiothreitol, 10 m*M* Tris-HCl, pH 7.4) and immediately submitted to the following experiments. Marker enzyme analysis was performed using 5′-nucleotidase as a marker for the plasma membrane (16). Protein was determined according to the method of Lowry *et al.* (17) using bovine serum albumin (BSA) as a standard. The specific activity of 5′-nucleotidase was increased 7-fold from 1.5 to 10.5 μmole/mg protein/hr in the plasma membrane fraction, when compared to the homogenate.

PtdIns Kinase Assay. PtdIns kinase activity was measured as [^{32}P]phosphate incorporation from [γ-^{32}P]ATP into PtdIns phosphate (PtdIns*P*) in the presence of PtdIns and Mg^{2+} (15). The assay buffer contained 100 m*M* Tris/HCl, pH 7.9, 1 m*M* EGTA, 10 μM [γ-^{32}P]ATP ($1.0–2.0 \times 10^{14}$ cpm/mol), 1 m*M* $MgCl_2$, 10 μ*M* PtdIns, membrane protein (5–20 μg protein) and agents to be tested in a total volume of 100 μl. PtdIns was added after dispersal by brief sonication. The reaction was started by the addition of membrane suspensions and was incubated at 37°C for desired interval time. The reactions were terminated by adding 0.5 ml of chloroform/methanol (1:2, v/v) and the phases separated with 0.1 ml chloroform and 0.1 ml of 10 m*M* ethylendiaminetetraacetic acid (EDTA). The upper phase was reextracted with preequilibrated lower phase, and combined with the lower phases. The extracted lipid was washed well with preequilibrated upper phase. PtdIns*P* was isolated by thin layer chromatography on silica gel 60 using a sequential ascending solvent system in chloforom/methanol/4 *M* NH_3 (9:7:2, v/v/v) flowed by chloroform/methanol/acetic acid/water (25:15:4:2, v/v/v/v) (18). Dried plates were autoradiographed to locarize PtdIns*P* by comparison with authentic PtdIns*P*. The areas corresponding to the standard was scraped and the radioactivity was counted. Most (>98%) of the radioactivity in the lipid fraction comigrated with the authentic PtdIns*P*. Under these conditions, less than 5% of ATP was consumed or degraded.

To identify phosphorylation position on the inositol ring of ^{32}P-labeled PtdIns*P*, the product was analyzed by deacylation and high performance liquid-chromatography analysis, as described previously (19). Approximately 95% of the radioactivity in the ^{32}P-PtdIns*P* was accounted for by PtdIns 4-*P* and less than 5% by PtdIns 3-*P*.

Membrane Protein Autophosphorylation Assay. Autophosphorylation was determined in membranes isolated as described above. The reaction mixture in a final volume of 100 μl contained 100 m*M* Tris/HCl, pH 7.9, 1 m*M* EGTA, 10 μ*M* [γ-^{32}P]ATP ($1.0–2.0 \times 10^{14}$ cpm / mol), 1 m*M* $MgCl_2$, membrane protein (5–20 μg protein) and desired agents. The reaction at 37°C for 5 min was initiated with membrane fractions, terminated by applying 50 μl of the reaction mixture into a solubilizing buffer (40 m*M* HEPES, 1% Triton X-100, 10% glycerol, pH 7.4). Then the lysates were centrifuged at 100,000*g* for 1 hr to remove insoluble fraction. The proteins in the resulting supernatant were precipitated with 10% trichloroacetic acid (TCA) and counted (20).

Statistics. Statistical analysis was performed by *t*-tests. Differences were considered significant if $P < 0.05$.

Materials. [γ-^{32}P]ATP (111 TBq/mmol) was obtained from New England Nuclear. Silica gel plates were purchased from Merck. Human prolactin (30 IU/mg), sheep prolactin (50 IU/mg), human placental lactogen, thrombin and estradiol-17β (E_2) were from Sigma, and all chemicals were of reagent grade.

Results

Effects of PRL on PtdIns Phosphorylation. Incubation of the plasma membranes isolated from endometrial fibroblasts with [γ-^{32}P]ATP and PtdIns caused [^{32}P]phosphate incorporation into PtdIns*P*, as shown in Figure 1. The level of PtdIns*P* increased over the first 2–3 min of incubation, attaining a steady-state level by 3–5 min that was maintained for at least 5 min (data not shown). The PtdIns phosphorylation was stimulated by vanadate ion in a dose-dependent manner; half-maximal enhancement occurred at approximately 50 μM vanadate (Fig. 2). This indicated the presence of vanadate-responsive PtdIns kinase in the plasma membrane from human endometrial fibroblasts.

PRL (1 μM) brought about a remarkable inhibition in the PtdIns kinase with respect to both basal and vanadate-responsive activities (Fig. 2). The maximal PtdIns*P* production rate was suppressed by approximately 80% from 30 pmol/mg protein/min to 6 pmol/mg protein/min, and the basal activity in the absence of vanadate ion was from 12 pmol/mg protein/min to 1.5 pmol/mg protein/min. The inhibitory action of PRL was dose dependent, and the half-maximal effect occurred at a concentration between 1 and 10 nM (Fig. 3). In contrast to PRL, some hormones targeting endometrial fibroblasts (thrombin and estradiol-17β) and lactogenic hormones (human placental lactogen and sheep PRL) had no effect on the membrane-associated PtdIns kinase activity, based on two preliminary experiments (data not shown).

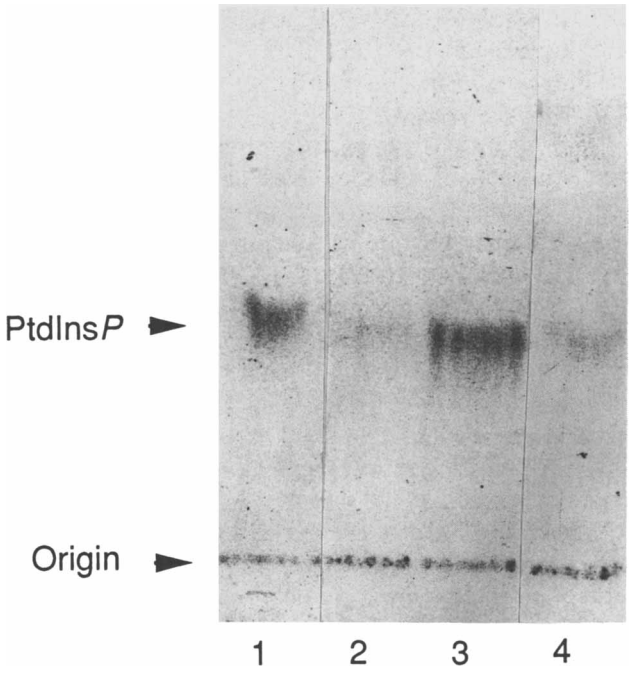

Figure 1. Effects of PRL and vanadate on PtdIns phosphorylation by plasma membrane prepared from endometrial fibroblasts. Plasma membranes (50 μg protein) were incubated for 5 min with [γ-^{32}P]ATP (10 μM) and PtdIns (10 μM) in the presence of vehicle (lane 1), PRL (1 μM, lane 2), vanadate (50 μM, lane 3) or PRL plus vanadate (lane 4). The extracted lipid was analyzed on thin layer chromatography, and bands were visualized by autoradiograph. Shown is a representative profile of six separate experiments which all gave similar results.

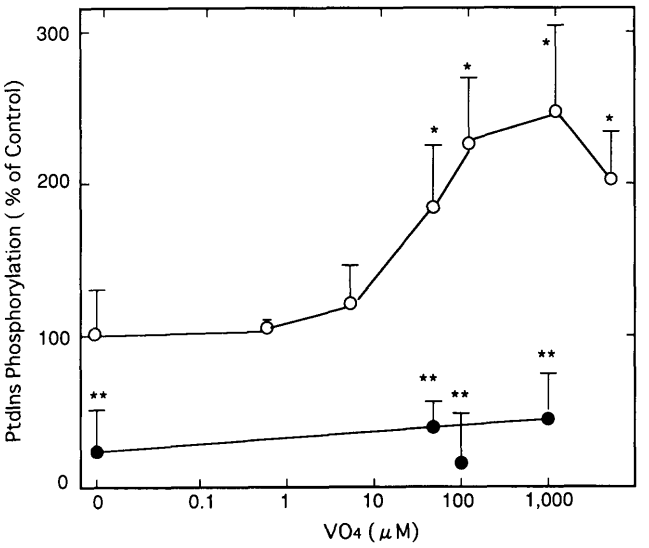

Figure 2. Effects of PRL on vanadate-responsive PtdIns kinase activity associated with plasma membrane isolated from endometrial fibroblasts. Plasma membranes (50 μg protein) were incubated for 5 min with [γ-^{32}P]ATP (10 μM) and PtdIns (10 μM) in the presence of various concentrations of vanadate ion alone (○) or vanadate ion plus PRL (1 μM) (●). The points represent the mean ± SD of three experiments and are expressed as percentages of control (neither PRL nor vanadate, 12 pmol/mg protein/min). $^*P < 0.01$ versus control. $^{**}P < 0.01$ versus no PRL at respective concentration of VO_4.

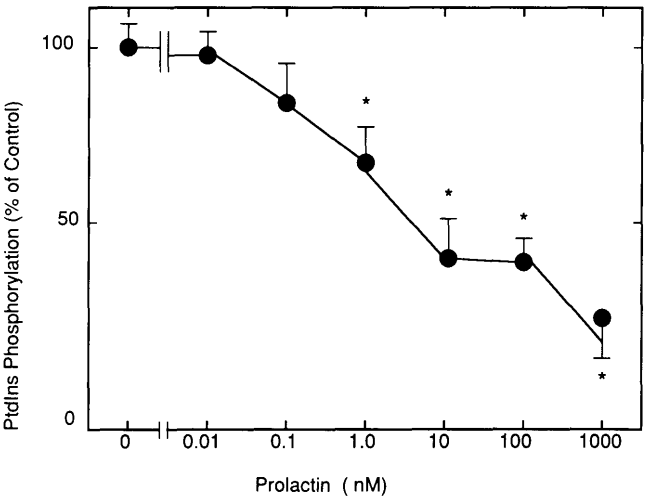

Figure 3. Effect of increasing doses of PRL on vanadate-responsive PtdIns kinase activity associated with plasma membrane isolated from endometrial fibroblasts. Plasma membranes (50 μg protein) were incubated for 5 min with [γ-^{32}P]ATP (10 μM), PtdIns (10 μM) and vanadate (100 μM) in the presence of various concentrations of PRL. The points represent the mean ± SD of three experiments and are expressed as percentages of control (no PRL, 28 pmol/mg protein/min). $^*P < 0.01$ versus control.

To determine whether the effects of PRL that was observed *in vitro* might occur *in vivo*, the endometrial fibroblasts were incubated in an HBSS with 1 μ*M* PRL for 5 min. PtdIns kinase activity measured in plasma membrane fraction isolated from these cells was 37 ± 12% (the mean ± SD of three experiments, $P < 0.01$) lower than those from control cells.

Effects of PRL on the Formed PtdIns Degradation. To demonstrate that the effect of PRL to decrease the rate of PtdIns phosphorylation by the plasma membrane fraction was not due to enhancement of its degradation, the loss of ^{32}P-prelabeled PtdIns*P* was measured under conditions identical with those used for assay of the kinase activity (Fig. 4). Plasma membranes were labeled with [γ-^{32}P]ATP for 5 min, and then incubated in buffer with 50 μ*M* ATP and with or without PRL. There was slow decrease of ^{32}P-labeled PtdIns*P* during the incubation in the presence or absence of PRL (1 μ*M*). PRL had no effect on the degradation of PtdIns*P*. Under these conditions of extremely low free Ca^{2+} concentration (the buffer contained 1 m*M* EGTA and no added Ca^{2+}), degradation is catalyzed almost exclusively by phosphomonoesterase but not by phospholipase C (21).

Effects of PRL on Autophosphorylation of Membrane Protein. When the plasma membrane fraction was incubated with [γ-^{32}P]ATP in the presence of Mg^{2+}, there was time-dependent incorporation of [^{32}P]phosphate into protein fraction, indicating the presence of both the endogenous protein substrates and the kinases in the membrane. As shown in Figure 5, vanadate ion (100 μ*M*) enhanced the protein phosphorylation. The vanadate-responsive and basal phosphorylation rate were attenuated to 25% by PRL (1 μ*M*).

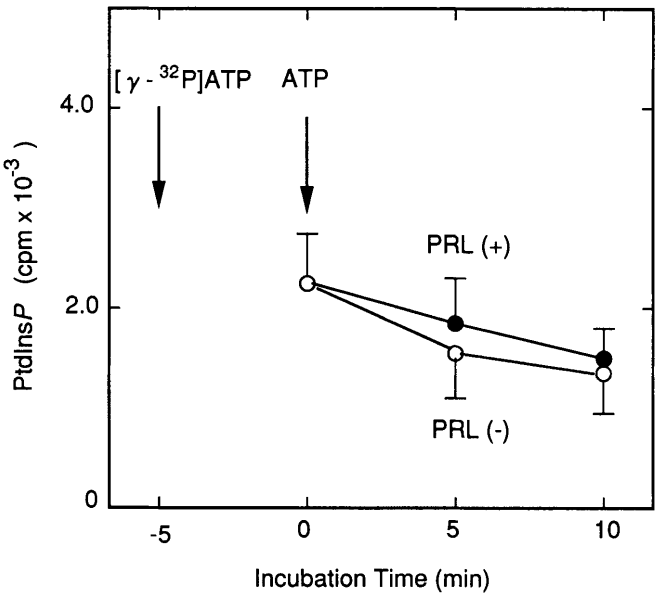

Figure 4. Lack of effect of PRL on degradation of PtdIns*P* formed by plasma membranes from endometrial fibroblasts. Plasma membranes from endometrial fibroblasts were preincubated with [γ-^{32}P]ATP (10 μ*M*) and PtdIns (10 μ*M*). After 5 min, the reactions were chased with ATP (50 μ*M*) in the absence (○) or presence (●) of PRL (1 μ*M*). The points represent the mean ± SD of two experiments.

Discussion

Carcinoma cells and stromal cells such as fibroblasts coregulate their proliferation *in vivo* and *in vitro* (22–28). Endometrial carcinomas, but not from benign tumor myoma or normal tissues, produce and secrete a putative growth-promoting peptide(s) for endometrial fibroblasts (29, 30), as well as certain other malignant tumors (31, 32). We have also found that PRL causes a marked inhibition on the mitogenicity of the extract (12). The present study extends our previous observations to demonstrate a possible mechanism for the antigrowth action of PRL.

The first step in the interaction of many growth factors with the fibroblasts is binding of the factors to specific receptors (1–4). The binding of the factors pro-

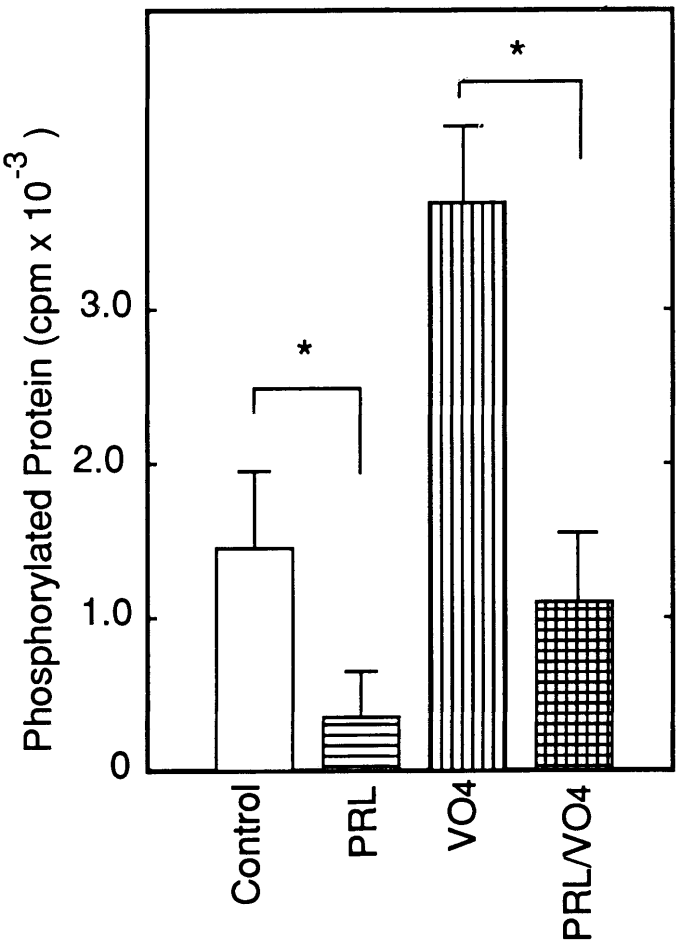

Figure 5. Effects of PRL and vanadate on autophosphorylation of plasma membrane prepared from endometrial fibroblasts. Plasma membranes (50 μg protein) were incubated for 5 min with [γ-^{32}P]ATP (10 μ*M*) in the presence of vehicle, PRL (1 μ*M*), vanadate (50 μ*M*) or PRL plus vanadate. The results are expressed as pmol ^{32}P incorporated/mg protein. The points represent the mean ± SD of three experiments. *$P < 0.01$.

motes the generation of early signals in the membrane, including stimulation of phosphoinositide metabolism. The membrane-receptor linked PtdIns kinase and/or tyrosine kinase is now considered to have functions in the mitogenic cellular responses. The putative growth factor in the endometrial carcinoma is yet not identified and purified, and we thereby hesitated to examine the ability of the growth factor to stimulate the PtdIns kinase activity in our system. However, exposure to vanadate ion, whose growth-promoting activity is well established (13, 14), can stimulate a tyrosine kinase-PtdIns kinase coupled system without any interaction with receptor on cell surface, much like those described for a number of growth factors and oncogene encoded products (2, 3, 8, 33). Our results presented here showed a simultaneous increase in PtdIns phosphorylation along with stimulation of protein autophosphorylation by vanadate in purified plasma membrane from human endometrial fibroblasts. The demonstration of vanadate-responsive PtdIns kinase activity in the isolated membranes might suggest an involvement of this enzyme, at least in part, in the initial transduction of the mitogenic signal in endometrial fibroblasts. PRL caused a marked noncompetitive inhibition on the vanadate ion-stimulated PtdIns kinase (Fig. 2) and protein kinase activities associated with plasma membranes (Fig. 5). A PtdIns kinase activity present in the plasma membrane was also decreased when the whole cells were incubated with PRL prior to disruption. It is important to note that our data do not definitively exclude the possibility that PRL stimulated-degradation of ATP served simply to provide deleted substrate for PtdIns kinase rather than suppressing the activity of the kinase. However, this explanation seems very unlikely because (i) less than 5% of the added ATP was consumed or degraded under our condition; and (ii) less than 15% of exogenous PtdIns was consumed. When explored the correlation between PRL inhibition of PtdIns kinase activity (Fig. 2) and mitogenic activity (26), the dose-response characteristics of these effects were very closed; half-maximal effects occurred with 1 to 10 n*M* PRL. We therefore concluded that PRL caused a decrease in the mitogenicity most likely as a result of the inhibition of a PtdIns kinase.

With regard to PRL action mechanism, protein kinase C is involved in the PRL stimulation of endometrial proliferation as well as in the PRL stimulation of mammary gland lactation processes (34), although little information is available on PRL transmembrane signaling. In this study, the PRL action, inhibition of PtdIns kinase, occurred in the plasma membrane from endometrial fibroblasts. These data made using highly enriched preparation of plasma membrane seems to constitute the most convincing evidence that the event in plasma membrane associated with PRL receptor is primarily stimulated during cell activation by PRL. Our finding might provide the alternative PRL signaling pathway independent on protein kinase C translocation, in the endometrial fibroblasts known to have specific receptor site (12). Assuming that, one may then inquire as to the mechanism for membrane metabolic events in response to PRL resulting in PtdIns kinase inhibition. Although there are several reports that address the regulation of PtdIns kinases *in vivo* (2, 3), the mechanism that regulate PtdIns kinases *in vitro* through PRL receptor is still unknown.

Lastly, carcinoma cells require cogrowth of supporting cells such as stromal cells for their own growth. PRL is synthesized by endometrial stroma (35) and binds to endometrial fibroblasts, and possess antigrowth activity against the mitogenic activity from the carcinoma (12). We demonstrated the inhibitory action of PRL on PtdIns kinase and protein autophosphorylation (perhaps through tyrosine kinase) associated with plasma membranes. It is widely accepted that activation of the membrane-receptor linked PtdIns kinase constitutes a likely early step in their mitogenic action. The antimitogenic action of PRL on human endometrial fibroblasts might be mediated through the inhibition of the PtdIns kinase. The new action of PRL can give a better insight as to the mechanism of carcinoma growth and invasion.

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