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Prolactin Stimulation of Phosphoinositide Metabolism in CHO Cells Stably Expressing the PRL Receptor

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PRL receptor (PRL-R) activation by PRL triggers a cascade of intracellular events including homodimerization of the receptor, activation of cytoplasmic receptor-associated tyrosine kinase and tyrosine-phosphorylation of various signal transducers. In CHO cells, transfected with the long form of PRL-R, an increase in [Ca^{2+}]i was observed following PRL stimulation whereas Ca^{2+} is generally coupled with the phosphoinositide metabolism. In this study, we investigated phosphoinositide involvement in the PRL transduction pathway. We report that PRL induces rapid increases in two novel inositol phospholipids, almost certainly PtdIns(4,5)P2 and PtdIns(3,4,5)P3. Pre-traitment of CHO cells with wortmanin, a specific PtdIns3-kinase inhibitor, considerably reduces the PRL-induced increase in PtdInd(3,4,5)P3, thus suggesting an involvement of this enzyme in the cascade of activation of cytoplasmic kinase proteins. A pathway beginning with the activation of PtdIns3-kinase, phosphorylation of PtdIns(4,5)P2 and rapid synthesis of PtdIns(3,4,5)P3 is proposed. PtdIns(3,4,5)P3 may acts as a lipid second messenger, directly or indirectly responsible for some of the multiple cell changes attributed to PRL.

Prolactin receptors (PRL-R) belong to a recently recognized family including receptors for growth hormone (GH) and cytokines (1, 2). PRL-R do not contain a tyrosine kinase domain but are associated with cytoplasmic tyrosine kinase of the JAK family (JAK2) (3,4). Most studies performed on the PRL transduction pathway have demonstrated that activation of PRL-R triggers a cascade of events including receptor dimerization (5), JAK2 kinase activation followed by tyrosine-phosphorylation of cytoplasmic signal transducers such as STATs proteins (6,7), protein-tyrosine kinases (8) MAP kinases (9), and PRL-R itself (10).

The signal transduction mechanism for the full-length PRL receptor has been studied using a CHO (Chinese hamster Ovary) line stably expressing PRL-R. The long form of the rabbit mammary gland PRL receptor cDNA, when transfected into CHO cells induces functional PRL receptors (11). In the CHO cell line, we have shown that, in addition to the events reported above, PRL induced both Ca^{2+} mobilization from intracellular stores and Ca^{2+} influx (12). The mechanisms responsible for the Ca^{2+} mobilization and entrance are not yet known. Over the last two decades, Ca^{2+}, an essential link in many agonist-evoked responses, had generally seemed to be coupled with the phosphoinositide metabolism (13). In a variety of cell types, PRL stimulates the generation of 1,2 diacylglycerol and protein kinase C and but without affecting inositol trisphosphate production. Thus the effect of PRL stimulation on Ca^{2+} homeostasis seems to be independent of Ins(1,4,5)P3. These data led us to the hypothesis that other inositol polyphosphate isomers may be involved in the PRL transduction pathway. Evidence has accumulated for a role of Ins(1,3,4,5)P4, InsP5 and InsP6 in some transduction pathways. Furthermore, Ins(1,3,4,5)P4 can apparently mobilise intracellular Ca^{2+} stores in cerebellar and adrenal microsomes (14). Ins(1,3,4,5)P4 has also been shown to open Ca^{2+} channels (15).

In this study, we investigated a putative effect of PRL on the phosphoinositide metabolism. Our results show that PRL stimulates a rapid, transient accumulation of PtdIns(4,5)P2 and PtdIns(3,4,5)P3.

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Abbreviations: Ins. inositol; InsPs. inositol phosphates; InsP1. inositol monophosphate; InsP2. inositol bisphosphate; Ins(1,3,4)P3. Inositol 1,3,4-trisphosphate; Ins(1,4,5)P3. inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P4. inositol 1,3,4,5-tetrakisphosphate; InsP5. inositol pentakisphosphate; InsP6. inositol hexakisphosphate; PtdIns. phosphatidylinositol; PtdIns4P. phosphatidylinositol 4-monophosphate; PtdInsP2. phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P3. phosphatidylinositol 3,4,5-trisphosphate.

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Further, we studied the role of PtdIns3-kinase in the accumulation of these highly phosphorylated inositol lipids, PtdIns(3,4,5)P3 being a physiologically important by-product of PtdIns3-kinase (16). Wortmannin, a specific, potent inhibitor of PtdIns3-kinase, prevented PRL stimulation of PtdIns(3,4,5)P3 accumulation. These data are consistent with recent observations showing the activation of PtdIns3-kinase by PRL in Nb2 cells (17).

EXPERIMENTAL PROCEDURES

Cell cultures. CHO cells were transfected with the long form of PRL receptor cDNA (CHO-E32) as previously described (18). The cells were grown in Ham's F12 medium (Seromed, Strasbourg, France) supplemented with 10% (v/v) fetal calf serum (Gibco, Grand Island, NY) L-glutamine (2.5 nM, Sigma) and 1% penicillin-streptomycin (Gibco). The cells were cultured in petri dishes (10 cm, Nunc Polylabo, Strasbourg, France) and used 5 days after seeding. At this time, cell density was appoximately 6×10^6 cells/petri dish. The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Labelling of inositol lipids. The cells were incubated for 2 days in serum-free medium containing 10 μCi/ml of myo[2-^{3}H]-inositol (specific radioactivity: 10-20 Ci /mmol, ICN, USA) at 37°C. At the end of labelling, following removal of labelled media, cells were washed for 1 min with myo[^{3}H]-inositol-free medium before experimental treatment.

Extraction and separation of inositol lipids. The cells were stimulated with PRL 10 nM for various lengths of time. At the end of stimulation, the reaction was stopped by addition of 1 ml methanol. Cells were removed from petri dishes with a rubber scraper, transferred to 16×125-mm glass tubes and rinsed again with 1 ml methanol. Inositol lipids were extracted as previously described (19). Chloroform (2ml) was then added to each sample and the mixture was sonicated for 30 sec, vortexed, then sonicated again for 2 min. KCl 0.9% (0.5 ml) was added to the mixture, then centrifuged at 6,000g for 5-min. The upper aqueous phase was discarded and the lower chloroformic phase washed again with 500 μl KCl 0.9%. It was then centrifuged again at 6,000g for 5-min. The final chloroformic phase was evaporated to dryness, and the lipid pellet redissolved in 200 μl hexane. Samples were stored at −20°C until thin layer chromatographic (TLC) separation.

Silica Gel 60 plates (20×20 cm; 0.5 mm, Merck, Darmstadt RFA) were preoxalated with a mixture of potassium oxalate 1%, 1M ethanol 50% and 1mM EDTA, then allowed to dry overnight under a continuous nitrogen flow. Inositol lipids and lipid standards were streaked and then separated as previously described (20) using a mixture of methanol/ chloroform/ ammonium hydroxyde/ water (100: 70: 15: 25; v/v/v/v). Phospholipid standards (phosphatidic acid, PtdIns, Ptd4InsP, and PtdIns4,5P2) and lipid pellets were streaked in a 3.5 cm stripes on the plates. After migration, phospholipid standards and labelled lipids were revealed with 2,7-dichlorofluorescein. Labelled lipids were scraped from the plates into tubes containing 3ml scintillation liquid for radioactivity counting.

Alkaline hydrolysis of membrane inositol lipids. Inositol lipids were prepared as described earlier. Stripes from silica plates containing the separated inositol polyphospholipids were scraped and the silica was recovered in glass tubes. The lipids were extracted using a chloroform-methanol-HCl (2/1/0.02 : V/V/V) mixture. This mixture was vortexed for 30 sec and centrifuged (3000g, 5 min). The upper phase was discarded, the pelleted silica remained at the bottom of the vial, the chloroform phase was recovered and evaporated under nitrogen flow.

Each sample was treated for 1 h with 300 μl of 2N KOH. The hydrolysis reaction was stopped by adjusting the pH to 7-7.2 with a 70 % solution of perchloric acid. The KCl precipitate was removed by centrifugation (3000 g, 5 min). Each supernatant was stored at −20°C until subsequent analysis by HPLC after addition of unlabelled InsPs as carriers. InsPs was separated by HPLC, as previously published (21, 22, 23), using a Partisil 10-Sax Interchrom (25*0,46 cm, 10μm from Interchim, Montluçon, France) anion-exchange column. InsPs were eluted by increasing the concentration of ammonium phosphate (pH 3.7, adjusted with orthophosphoric acid) from 0 to 2 M at a rate of 1.3 ml/min. Radioactivity was continuously recorded using a radiodetector (Flow-one 500TR Packard) and 3 ml scintillation liquid (quick szint 306 zinsser) per 1ml eluant.

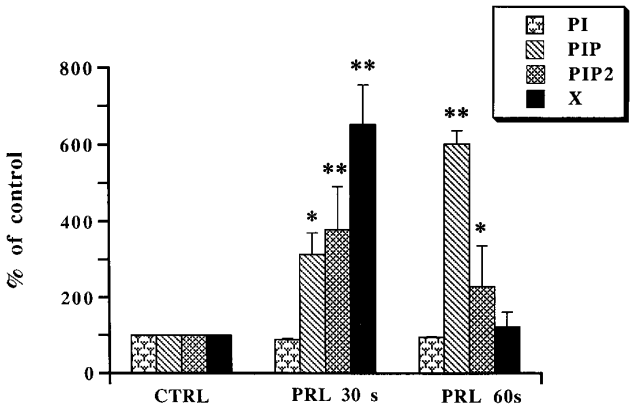

FIG. 1. Effects of PRL stimulation (10 nM) on phosphoinositide turnover in CHO cells. Inositol lipids were extracted from CHO cells and separated using thin layer chromatography on preoxalated silica Gel plates in methanol/ chloroform/ ammonium hydroxyde/ water (100: 70: 15: 25; v/ v/ v/ v). After co-elution, phospholipid standards and labelled phosphoinositides were revealed with 2,7-dichlorofluorescein. Labelled phosphoinositides were scraped for counting. Values are expressed in % of control values (mean ± SEM). PtdIns (PI), PtdIns4P (PIP), PtdIns(4,5)P2 (PIP2), and X, a highly-phosphorylated phosphoinositide, were separated.

Statistical analysis. The data were analysed using the Student's *t* -test for unpaired samples to determine significant differences between control and treated groups. The data were presented as the mean ± SEM expressed in % of control values.

Materials. Ovine PRL (oPRL-19) was kindly provided by the NIDDK (National Hormone and Pituitary Program, University of Maryland School of Medicine, Baltimore, MD). It was used at 10 nM.

Phosphoinositol lipids used as standards were obtained from Sigma. Tritiated InsPs standards (InsP1, Ins(1,3)P2, Ins(1,4)P2, Ins(1,4,5)P3, Ins(1,3,4,5)P4, InsP6) were purchased from NEN (Dupont de Nemours, les Ulis, France). Ins(1,3,4)P3, Ins(1,3,4,6)P4 and Ins(1,3,4,5,6)P5 were laboratory made.

Wortmannin (Sigma, France) was stored in dimethyl sulfoxide at −80 °C and diluted into the culture medium prior to use.

RESULTS AND DISCUSSION

The phosphoinositides synthetized by myo-[^{3}H]inositol-labeled CHO cells were analysed at 30 and 60 sec. In controls and stimulated groups (n=6), PtdIns, PtdIns4P, PtdIns4,5P2, co-eluted with standards, and a highly phosphorylated phosphoinositide (X in Fig 1) were separated. PRL decreased the level of PtdIns whereas polyphosphorylated inositol lipids increased

after incubation for 30 sec and 60 sec with PRL. In control cells, the PtdIns(4,5)P2 level was only 0.61 % of total labeled lipids. It increased rapidly following PRL (10 nM) administration to 2.31% at 30 sec and returned to 1.40 % at 60 sec. Another phosphorylated inositol lipid, X, also increased in response to PRL stimulation from 0.34 % to 2.21 % at 30 sec. The increase was transient since it returned to 0.42 % at 60 sec. Conversely, PtdIns decreased from 96.96 % to 86.05 % 30 sec after PRL administration. These data were expressed in % of control values as shown in Fig. 1.

Another similar separation procedure was carried out to identify X. Extracted products were submitted to alkaline hydrolysis to turn PIns into InsPs as described in the *experimental procedure section*. These hydrolyzed products were then analysed by liquid chromatography (anion-exchange HPLC). The presence of PtdIns, PtdIns4P, PtdIns(4,5)P2 and an inositol lipid more highly phosphorylated than PtdInsP2 was validated by comparing InsPs obtained after lipid hydrolysis to the various standards separated under similar elution profiles (Fig.2). InsP1 was separated after PtdIns hydrolysis while InsP1 and Ins(1,4)P2 were obtained following PtdInsP hydrolysis. PtdIns(4,5)P2 hydrolysis produced InsP1, Ins1,4P2 and Ins(1,4,5)P3. The presence of these InsPs proved that PtdIns, PtdIns4P or PtdIns(4,5)P2, respectively, were present prior to hydrolysis.

A radioactive peak, corresponding to Ins(1,3,4,5)P4 was observed when examining the "X"-stripe hydrolyse products, showing that X is a PtdIns(3,4,5)P3 (Fig.2a). These results indicate that PRL induces a rapid increase in PtdIns(4,5)P2 and PtdIns(3,4,5)P3 production in CHO cells. The production of PtdIns(3,4,5)P3 was consistent with other studies: indeed, highly polar inositol lipids appear in many other cells in response to a range of growth factors and activated oncogenes (24, 25, 26). This rapid, transient PtdIns(3,4,5)P3 production can be explained by PtdIns(4,5)P2 phosphorylation, induced by a PtdIns3-kinase since this kinase is already known to be involved in GH, IL2, EPO transduction processes (27, 28, 29). Activation of PtdIns3-kinase by PRL has also been recently demonstrated in Nb2 cells (17). Indeed, this enzyme contains two SH2 (Src homology 2)-domains and a catalytic subunit (30) found in association with a variety of receptor and non-receptor protein-tyrosine kinases.

To check the direct involvement of PtdIns3-kinase activation in PRL transduction pathway leading to PtdIns(3,4,5)P3 synthesis, we incubated CHO cells for 30 min with wortmannin (150 nM), a PtdIns3-kinase inhibitor, prior to PRL stimulation (Fig.3). As expected, PtdIns(4,5)P2 and PtdIns(3,4,5)P3 levels increased after PRL stimulation of CHO cells (about 5 and 5.5-fold respectively). In pre-treated cells, however, wortmanin treatment did not significantly affect

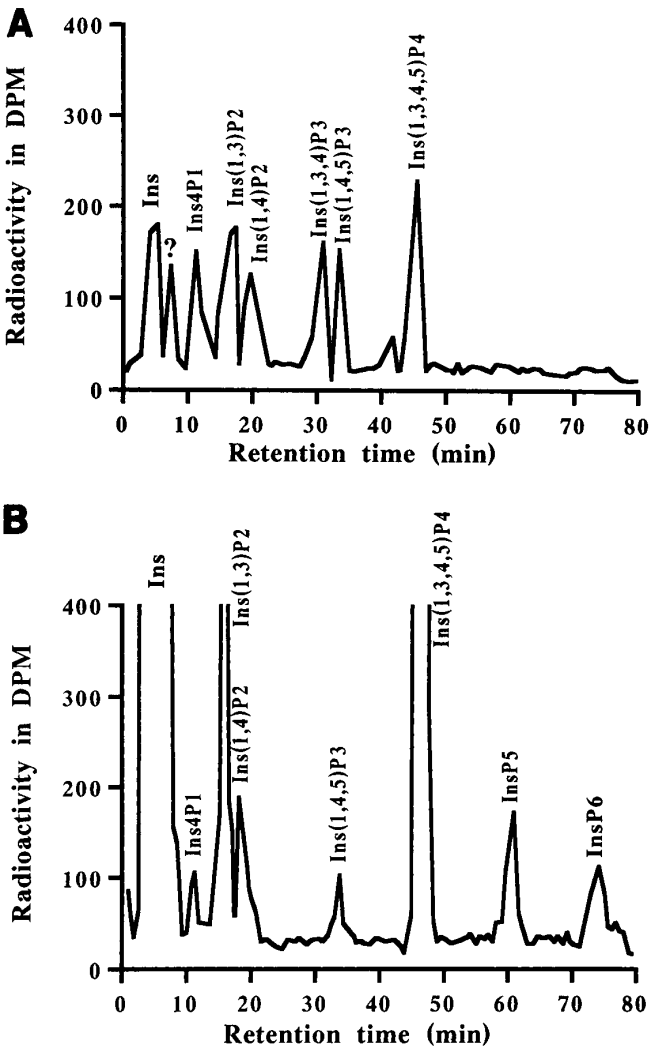

FIG. 2. Separation by anion-exchange HPLC of labelled InsPs extracted from "X" stripe alkaline hydrolysis (A) and labelled standards (B) The elution was achieved by increasing the concentration of ammonium phosphate buffer from 0 to 2M at a rate of 1.3 ml/min. Elution profiles of both groups were compared. The retention times of InsPs standards allowed identification of InsPs peaks. Peaks that co-eluted with the various standards were assumed to have the same identities as the standards.

PtdIns(4,5)P2 synthesis while PtdIns(3,4,5)P3 production was considerably reduced.

In the present study, we demonstrated, on the one hand, that PRL increases PtdIns(3,4,5)P3 production and, on the other hand, that wortmaninn inhibits the PRL-mediated PtdIns(3,4,5)P3 increase. In a variety of cell types, a number of intracellular kinases including PtdIns3-kinase are stimulated following tyrosine phosphorylation of cytokine receptors (28, 29). PtdIns3-kinase activation upon GH action has been demonstrated in rat adipocytes. GH stimulation induced rapid, dose-dependent tyrosine-phosphorylation of insulin-receptor substrate 1 (IRS1) and its association with the p85

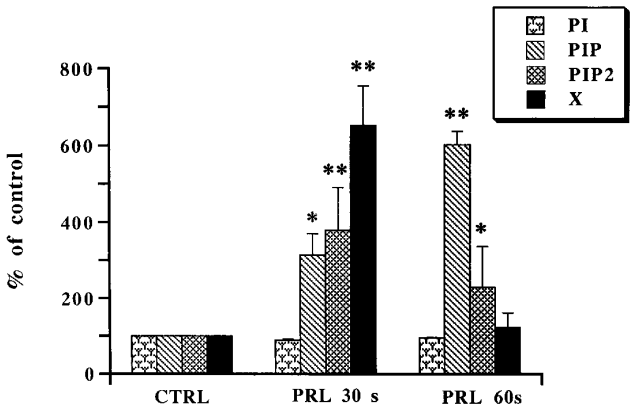

FIG. 3. Effects of Wortmaninn on PRL-activated polyphosphoinositide production. The cells were incubated with and without wortmaninn (150 nM) for 30 min prior to PRL stimilation (10nM, 30 sec). Inositol lipids were then extracted and separated by TLC, as described in experimental procedures. Only PtdIns(4,5)P2 and PtdIns(3,4,5)P3 levels were represented and expressed in % of control values (mean ±SEM).

subunit of PtdIns3-kinase (27). These insulin effects of GH in rat adipocytes are blocked by wortmannin (31). The activation of PtdIns3-kinase by PRL has been recently demonstrated in Nb2 lymphoma cells (17). Our results thus confirm and somewhat extend these observations though PRL-R of Nb2 cells are able to recognize GH and are clearly involved in the proliferation process which is not the case in CHO cells.

The exact role of these heavily phosphorylated inositol lipids and PtdIns3-kinase in intracellular signalling is not well known and may differ from one cell type to another. They are known to be potential sources of highly phosphorylated inositol phosphates such as InsP4, InsP5, InsP6. The latter may act as second messengers, as has already been reported in recent studies (14, 15, 32). We do not know, however, how PtdIns(3,4,5)P3 is metabolized in CHO cells and, to our knowledge, a metabolic pathway from PtdIns(3,4,5)P3 to Ins(1,3,4,5)P4 has not yet been described.

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