Prolactin-induced Cell Proliferation in PC12 Cells Depends on JNK but Not ERK Activation*

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The effects of pituitary and extrapituitary prolactin include cellular proliferation and differentiation. PC12 cells was used as a model to delineate respective signaling of prolactin. Prolactin acted as a mitogen for undifferentiated PC12 cells, as measured by significant increases in bromodeoxyuridine incorporation and in cell numbers, with an efficacy equal to epidermal growth factor. Both the long and short form of the prolactin receptor was expressed, yet only the long isoform was tyrosine-phosphorylated upon agonist binding. Functional prolactin receptor signaling was further demonstrated in the activation of JAK2 and phosphorylation activation of the transcription factors Stat1, -3, and -5a. Surprisingly, prolactin stimulated a sustained activation of Raf-B, without activation of the MAP kinases ERK1 or -2. Instead, in solid phase kinase assays using a glutathione S-transferase-c-Jun fusion protein (amino acids 1–79) as the substrate, a significant activation of the mitogen-activated protein Janus kinase (c-Jun N-terminal kinase; JNK) was observed. The prolactin-induced activation of JNK was prolonged and accompanied by a significant increase in c-Jun mRNA abundance and c-Jun protein synthesis. Moreover, analysis of bromodeoxyuridine incorporation at the single cell level revealed that epidermal growth factor-dependent incorporation was inhibited by PD98059 and independent of SB203580, whereas prolactin-induced incorporation was ERK and mitogen-activated protein kinase p38 independent but was abolished with JNK inhibition by 30 μM SB203580. Our studies suggest that prolactin may have a role in the growth of PC12 cells, where it stimulates concurrent mitogenic and differentiation-promoting signaling pathways.

Prolactin (PRL),1 which was originally identified as an anterior pituitary hormone involved in osmoregulation, reproduction, and behavior (1), has recently been shown to be a growth factor for many cell types in the developing and adult organism (1, 2). The recent emphasis on extrapituitary prolactin has underlined the role of prolactin as a neurotrophic factor. Furthermore, several vertebrate neuroendocrine tissues, including the central nervous system, peripheral neural populations, and adrenal, have been shown to express mRNA encoding the long and short forms of the prolactin receptor (PRLR). The study of prolactin action in the nervous system has been focused on the modulation of neuronal neurotransmitter expression and secretion in postnatal postmitotic neurons. However, the direct effects of prolactin in developing neural cells and the signal transduction pathways that mediate them have only recently been addressed (3, 4).

Moreover, the exact signal transduction pathways for the diverse biological actions of prolactin are little understood. The PRLR belongs to the superfamily of cytokine class-1 receptors (5). The PRLR, like the other members of this family, does not contain a tyrosine kinase domain but signals through activation of associated cytoplasmic tyrosine kinases of the Janus kinase (JAK) and Src kinase families (6–10). Prolactin binding promotes dimerization of PRLR, which triggers activation of tyrosine kinases and further transduction of the signal (8, 10, 11). The major PRLR-associated tyrosine kinase is JAK2 (7, 8, 12, 13). Association of JAK2 with a PRL-bound receptor is sufficient for activation of the kinase, phosphorylation of PRLR, and the recruitment and activation of members of the signal transducers and activators of transcription (STAT) family of transcription factors, in particular Stat1, Stat3, and Stat5 (14–16), which are considered as major effectors for prolactin-dependent cell proliferation and gene activation (17). In addition, phospholipase C-protein kinase C and the ERK and p38 MAP kinase activation have also been reported as effectors of PRLR (9, 18, 19). However, it is not clear whether these interacting or often parallel pathways specifically mediate cell proliferation or differentiation. With this background, we undertook these studies to investigate the potential role of prolactin as a growth factor in PC12 cells, a model system to delineate proliferation and differentiation signal transduction pathways.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium, epidermal growth factor (EGF), and calf serum were purchased from Life Technologies, Inc. Ovine prolactin was purchased from Sigma, and rat prolactin was provided by the National Hormone and Pituitary Program, National Institutes of Health. Polyclonal antibodies against Stat3, Stat5a, c-Jun, and JNK were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); a monoclonal antibody against ERK1/2, monoclonal antibody 4G10 against phosphotyrosine, a polyclonal antibody to JAK2, a polyclonal antibody to Raf-B, and recombinant protein A-agarose were purchased from Upstate Biotechnology, Inc.; the U5 monoclonal antibody against the prolactin receptor was from ABX Inc.; a monoclonal antibody to Stat1 was from Transduction Laboratories; rabbit anti-mouse IgG was from Jackson Laboratories; and anti-5-bromo-2′-deoxyuridine (BrdUrd) staining kit was from Roche Molecular Biochemicals. PP1, PD98059, and SB203580 were purchased from Alexis, Inc.

Cell Culture—PC12 cells, obtained from Dr. Aaron Fox (University of Chicago, MC 5058, WCH C-576, Chicago, IL 60637-1470. Tel.: 773-702-1136; Fax: 773-702-9234; E-mail: dm36@midway.uchicago.edu.
Cell Counting and Time Lapse Photography—PC12 cells were plated on Corning plates engraved with a 2 × 2-mm grid. Ten fields per dish of four per experimental group were chosen randomly, charted on the grid, and followed every 12 h by time lapse photography through a 20× objective on a Nikon Diaphot inverted microscope with a 20×, 0.40 NA objective and an 125 ASA Plus-X pan Kodak film. Cell numbers were then counted on black and white pictures. Sister cultures were trypsinized, and cell numbers were directly assessed with a Coulter counter (21).

Immunodetection of BrdUrd Incorporation—Briefly, PC12 cells were changed to Neurobasal plus N2 medium 36 h prior to indicated treatments. After 16 h of agonist treatment, BrdUrd (10 μM) was added for 60 min, and cultures were fixed and stained with anti-BrdUrd according to the manufacturer’s instructions with some modifications (4). The antibody binding was visualized with an anti-mouse alkaline phosphatase-conjugated IgG. Before mounting, nuclei were stained with bis-benzimide (Hoechst 33258, Sigma) to identify normal (intact) and apoptotic (fragmented) nuclei. Cells were viewed and photographed first for BrdUrd uptake under light using a Nikon 6000E camera and a ×20 lens. Assessment of cell numbers and apoptosis was done by switching to a UV optical filter and counting intact and fragmented nuclei.

Immunoblot Analysis—Total cell lysates, PC12 cells were lysed in RIPA buffer, i.e., a 10 mm Tris-HCl, pH 7.4, buffer containing 50 mm NaCl, 5 mm EDTA, 50 mm NaF, 1 mm Na3VO4, and 1% Triton X-100 with two protease inhibitors, leupeptin and EpSp59, at 1 μg/ml. After a 10-min centrifugation, supernatant solutions were assayed for protein content. Lysates normalized per protein content were analyzed with SDS-PAGE. The resolved proteins were transferred onto Immobilon membranes as described (22). To assure equal loading, membranes were first stained with the reversible dye Ponceau S. Abundance and electrophoretic ability of Raf-B, ERK1 and -2, and c-Jun in the various groups were detected by blotting the membranes with a monoclonal antibody. Immunoreactivity was visualized by further incubation with species-specific, horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence.

Immunoprecipitations—Immunoprecipitations were performed as described previously (7, 23). Cells were harvested in 500 μl of lysis buffer: 50 mm Tris-HCl, pH 7.5, 5 mm EDTA, 50 mm NaCl, 50 mm MgCl2, 1 mM thio-β-D-galactopyranoside, 1% Triton X-100 with two protease inhibitors, leupeptin and EpSp59, at 1 μg/ml. After a 10 min centrifugation, supernatant solutions were assayed for protein content. Lysates normalized per protein content were analyzed with SDS-PAGE. The resolved proteins were transferred onto Immobilon membranes as described (22). To assure equal loading, membranes were first stained with the reversible dye Ponceau S. Abundance and electrophoretic ability of Raf-B, ERK1 and -2, and c-Jun in the various groups were detected by blotting the membranes with a monoclonal antibody. Immunoreactivity was visualized by further incubation with species-specific, horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence.

RESULTS

Prolactin Stimulates Cell Proliferation in PC12 Cells—We used three different methods to study the effects of prolactin on the proliferation or differentiation of PC12 cells, namely cell counts, BrdUrd uptake, and time lapse photography. First, PC12 cells were grown for 2 days after plating and then arrested with preincubation in a chemically defined medium for 48 h or in Neurobasal medium plus N2 supplement. Under these conditions, proliferation ceases and cells acquire differentiated features (20, 26). At the end of this period, rat prolactin (1 μM) or vehicle was added for an additional time with calf serum (10%) and EGF (50 ng/ml) used in parallel cultures as positive mitogenic controls. After treatment, cells were trypsinized, and cell numbers were assessed directly with a Coulter counter. Prolactin was a mitogen, as strong as EGF, while calf serum was, as expected, stronger than both (Fig. 1A). Specifically, cell numbers after 2 days of treatment with prolactin (1 μM) had increased to an average of 5.45 × 105 ± 0.63 cells/dish versus 3.02 × 105 ± 0.15 in cultures treated with vehicle. The difference was statistically significant (p < 0.002), suggesting that PRL had induced mitosis. By 72 h, the difference in growth rate between control and prolactin-treated cells was not as dramatic; however, cell numbers in the prolactin-treated group remained significantly higher that in controls (p < 0.01). The increase in cell number was due to cell proliferation, as verified with BrdUrd incorporation studies. A multifold increase in the numbers of nuclei BrdUrd-positive nuclei was evident after prolactin stimulation as compared with controls (3.5-fold). This increase in the numbers of nuclei BrdUrd-positive nuclei was evident after prolactin stimulation as compared with controls (3.5-fold). This increase in the numbers of nuclei BrdUrd-positive nuclei was evident after prolactin stimulation as compared with controls (3.5-fold). This increase in the numbers of nuclei BrdUrd-positive nuclei was evident after prolactin stimulation as compared with controls (3.5-fold). This increase in the numbers of nuclei BrdUrd-positive nuclei was evident after prolactin stimulation as compared with controls (3.5-fold). This increase in the numbers of nuclei BrdUrd-positive nuclei was evident after prolactin stimulation as compared with controls (3.5-fold). This increase in the numbers of nuclei BrdUrd-positive nuclei was evident after prolactin stimulation as compared with controls (3.5-fold). This increase in the numbers of nuclei BrdUrd-positive nuclei was evident after prolactin stimulation as compared with controls (3.5-fold). This increase in the numbers of nuclei BrdUrd-positive nuclei was evident after prolactin stimulation as compared with controls (3.5-fold).
served with EGF (lower panel). This type of analysis also allowed observations on the state of differentiation and cell loss. Control cultures continued to differentiate during the elapsed 24-h time period, as evident from the increasing length of neurites (Fig. 2B, arrowheads in the upper panel). In the prolactin-treated cultures, several cells had completed a cell cycle over the same period of 24 h (Fig. 2B, arrows in the middle panel) and then assumed a polarized phenotype. In the EGF-treated cultures, while the number of proliferating cells appeared to be similar, the cells appeared to lag behind in cell cycle completion as compared with prolactin, with several of them observed in anaphase (Fig. 2B, lower panel, arrows). This time-lapse analysis also verified that cell loss was minimal (asterisk). All subsequent signaling studies were performed with the same experimental design to assure examination of the events within a time frame of what appeared as a concomitant signal for proliferation and differentiation by prolactin.

The Prolactin Receptor, JAK2, and STATs are Tyrosine-phosphorylated Acutely after Prolactin Treatment in PC12 Cells—Western blot analysis with the monoclonal anti-phosphotyrosine antibody 4G10 has established functional expression of PRLR by showing that several proteins were modified with time of PRL exposure (27). The long isoform of the prolactin receptor is one of the proteins modified by tyrosine phosphorylation in response to prolactin; JAK2 is then activated, and the PRLR-JAK2 complex propagates the signaling through recruitment of a member of the STAT transcription factor family. In order to establish activation of this pathway in PC12 cells, we employed immunoprecipitation combined with Western blot analysis of total lysates of prolactin-treated cells. First, cell lysates were immunoprecipitated with the U5 anti-prolactin receptor antibody (IP:U5) or the 4G10 anti-phosphotyrosine antibody (B); antibody binding was visualized using chemiluminescence. The long and the short isoforms of the receptor and the associate kinase JAK2 are indicated with arrows. The molecular mass of prestained markers is indicated with bars in kilodaltons. C, immunoprecipitation with a monoclonal antibody to Stat1 and Western blotting with 4G10 revealed the sustained tyrosine phosphorylation/activation of the transcription factor with time of exposure to prolactin. D, similar analysis of the Stat3 tyrosine phosphoryse content showed a less sustained increase with time of PRL treatment; the increases in tyrosine phosphorylation at 5 min were not affected by 1 μM SB203580, SB 30 μM, or 10 μM PPI. E, the Stat5a tyrosine phosphoryse profile in response to PRL is similar to that of Stat1 and Stat3, including the regulation by the indicated kinase inhibitors.

Fig. 2. Tyrosine phosphorylation of PRL receptor, JAK2, and Stat1, -3, and -5a in response to prolactin in PC12 cells. PC12 cells were incubated for times indicated with 1 nM prolactin. PRL receptors were immunoprecipitated with the U5 monoclonal antibody to PRL-R and immunoblotted with U5 (A) or the 4G10 anti-phosphotyrosine antibody (B); antibody binding was visualized using chemiluminescence. The long and the short isoforms of the receptor and the associated kinase JAK2 are indicated with arrows. The molecular mass of prestained markers is indicated with bars in kilodaltons. C, immunoprecipitation with a monoclonal antibody to Stat1 and Western blotting with 4G10 revealed the sustained tyrosine phosphorylation/activation of the transcription factor with time of exposure to prolactin. D, similar analysis of the Stat3 tyrosine phosphoryse content showed a less sustained increase with time of PRL treatment; the increases in tyrosine phosphorylation at 5 min were not affected by 1 μM SB203580, SB 30 μM, or 10 μM PPI. E, the Stat5a tyrosine phosphoryse profile in response to PRL is similar to that of Stat1 and Stat3, including the regulation by the indicated kinase inhibitors.
ning of these Western blots revealed that this protein co-migrated with a protein that was reactive to JAK 2 antibodies (data not shown), as anticipated.

To confirm activation of the JAK-STAT pathway by prolactin, the specific tyrosine phosphorylation of Stat1, -3, and -5a was investigated with immunoprecipitation of whole PC12 cell lysates with the respective antibodies and Western blotting with antiphosphotyrosine antibodies first and then reblotting with the immunoprecipitating antibodies. A rapid phosphorylation of Stat1 by prolactin became maximal at 15 min of exposure to prolactin. The phosphorylation/activation was sustained for hours and returned to unstimulated levels by 3 h (Fig. 2C). The time course of Stat3 (Fig. 2D) and Stat5 (Fig. 2E) tyrosine phosphorylation in response to prolactin was also acute and maximal by 15 min but slightly differed from that of Stat1 in that the tyrosine phosphorylation returned to basal levels in about 2 h. The regulation of the phosphorylation of all three STATs was also similar. As shown representatively in Fig. 2, D and E, low concentrations of SB203580, 1 μM (a concentration that only inhibits p38) or 30 μM (which inhibits p38, JNK2, and c-Raf), or Src family kinases (PP1, at 10 μM) had no significant effect on the PRL-induced tyrosine phosphorylation of these STATs.

Prolactin Activates Raf-B but Not ERK1 and -2 in PC12 Cells—Having demonstrated that prolactin is a mitogen in PC12 cells, we examined its coupling to ERK activation, the duration of which in PC12 cells is known to correlate with mitogenicity of the signal; i.e., EGF produces a relatively acute but transient activation of ERK 2, while NGF promotes a later but sustained activation of this kinase (28). In this prototypic mitogenic signaling pathway activated by EGF, the serine/threonine kinase Raf-B is upstream to the activation of the MAP kinases ERK1 and -2. Activated Raf phosphorylates and activates downstream kinases of the MEK family, thus initiating the MEK-MAP kinase cascade (29). Upon activation, Raf is phosphorylated on serine and threonine residues, and its apparent molecular weight increases (30). Therefore, activation of Raf-B in total lysates from PC12 cells treated for different times with prolactin was studied by Western blot analysis with anti-Raf-B antibodies (results of a representative Western experiment are shown in Fig. 3A). By 15 min of stimulation with prolactin, the mobility of Raf was obviously retarded, indicating activation of Raf molecules. The activation lasted for over 1 h. In comparison, the activation induced by EGF was more robust but less sustained (Fig. 3B). The effect of EGF, used as a positive control for activation, was established by 1 min, maximal by 5 min, and almost over by 15 min.

The time course of EGF-stimulated Raf phosphorylation was consistent with it being upstream to ERK activation, as seen in total lysates analyzed by Western blotting with a monoclonal antibody, which primarily recognizes ERK2 and cross-reacts with ERK1 (Fig. 3C). Because a significant number of amino acid residues are phosphorylated on ERK1/2 molecules when they are activated, the electrophoretic mobility of the activated molecules also changes. The result is the appearance of an additional band of slightly higher Mr (activated) right above the baseline band (nonactivated ERK) and the overall appearance of ERK1 or -2 as doublets bands in Western blots. Use of ERK mobility as a measure of its activation clearly showed that PRL did not cause detectable activation of ERK1 or -2 as concluded in several experiments (Fig. 3C), while EGF stimulated both ERK1 and ERK2 5 min after its introduction into the culture medium. The activation lasted several more minutes. ERK1 activation appeared more transient than that of ERK2, which lasted for 1 h. Similar experiments using different antibodies or in-gel assays (21, 31) also did not detect any ERK activation with prolactin, even with concentrations as high as 1 μM.

Prolactin Stimulates JNK Activity—The surprising finding that while Raf was activated by prolactin, several sensitive detection methods of ERK activation clearly showed that the prolactin receptor is not coupled to ERK1/2 in PC12 cells prompted us to investigate whether prolactin is coupled to activation of another major MAP kinase, the c-Jun N-terminal kinase (JNK). JNKs exclusively phosphorylate Ser63 and Ser73 in the transactivation domain of c-Jun, a component of the transcription factor AP-1, and we exploited this specificity to measure JNK activation in solid phase kinase assays. We first generated a fragment from amino acids 1–79 of c-Jun using PRL-stimulated PC12 cells as a template, as described under “Experimental Procedures.” A GST fusion c-Jun fragment was constructed and used as a substrate to assay JNK activation (Fig. 4). After different times of treatment with prolactin, JNK was immunoprecipitated with anti-JNK antibody and allowed to phosphorylate GST-c-Jun-(1–79) in vitro. The reaction products were analyzed by SDS-PAGE and autoradiography. Densitometry of the autoradiogram (a typical one is shown in Fig. 4A) revealed that JNK was activated by prolactin in PC12 cells. The activation had reached 3-fold over base line by 30 min and 10-fold, the maximal activation, by 1 h. The activation was sustained maximal for an additional 2 h until it declined to control levels after 6 h (Fig. 4A). This is the first demonstration of prolactin-receptor coupling to JNK in any cell type.

The activation of the transcription factor c-Jun downstream to prolactin receptor activation was verified in vivo as well. Total lysates from cells with different exposure time to prolactin were studied by Western blot analysis using a monoclonal anti-c-Jun antibody as described under “Experimental Procedures.” A significant increase in the synthesis of c-Jun was
Prolactin Activates JNK

Inhibition of JNK but Not ERK or p38 Activity Abolishes PRL-induced Proliferation—To further evaluate the role of JNK activation in the PRL-induced PC12 proliferation, we employed specific inhibitors of the three subclasses of MAP kinases, namely SB203580, which acts as a specific inhibitor of p38 at 1 \( \mu M \) and additionally inhibits JNK at concentrations over 10 \( \mu M \) (46), and PD98059 (50 \( \mu M \)) a specific inhibitor of MEK and its downstream ERKs (Fig. 5). PC12 cells were pretreated with vehicle (Fig. 5, upper row), 1 or 30 \( \mu M \) SB203580 (second and third row, respectively), or 50 \( \mu M \) PD98059 (bottom row) for 1 h prior to exposure to vehicle (left column) or the mitogenic stimulants PRL (middle column) and EGF (right column). BrdUrd incorporation as a measure of DNA synthesis and cell proliferation was assessed immunocytochemically 18 h later, as described under “Experimental Procedures.” Nuclear incorporation of BrdUrd in the vehicle group was minimal in all vehicle-treated cultures (left column) and with inhibition with 1 or 30 \( \mu M \) of SB203580 or PD98059 did not affect the basal mitotic activity in arrested cultures. One \( \mu M \) SB203580 and inhibition of p38 had no effect on the mitogenic activity of either PRL or EGF. Constatnt with the ERK activation result, MEK and ERK inhibition abolished the EGF-induced cell proliferation but had no effect on the PRL mitogenic activity (Fig. 5). Most importantly, inhibition of p38, JNK2, and c-Raf with 30 \( \mu M \) SB203580 had opposing actions, with no significant change on the number of BrdUrd-positive nuclei in the EGF-treated cultures but totally blocking the PRL-induced BrdUrd incorporation (Fig. 5).

DISCUSSION

In this study, we show that prolactin supports concurrent proliferation and differentiation of PC12 cells and present evidence that the PRLR-dependent activation of JNK is associated with the induction of one cell cycle.

Prolactin was mitogenic to PC12 cells. The proliferation rate, similar to that observed with EGF stimulation, caused the cell population to almost double before becoming quiescent. However, after 24 h of treatment, more cells appeared to be in anaphase in the EGF-treated cultures than in prolactin-treated cultures. In contrast, cells treated with prolactin appeared to have completed the cell cycle and to have assumed polarized morphology with longer processes (Fig. 1B). This finding suggests that pituitary and extrapituitary prolactin may be one of the agonists that participates in the expansion of the chromaffin cell population.

Prolactin Activates the JAK-STAT Pathway in PC12 Cells—Immunoprecipitation with U5 antibody showed that both the long and short PRLR isoforms are expressed in PC12 cells (Fig. 2). While both PRLR isoforms were detected in immunoprecipitations with the U5 monoclonal antibody, only the long form was tyrosine-phosphorylated in response to prolactin. The U5 anti-PRLR antibody co-immunoprecipitated JAK2 as previously reported (7, 8, 13). The time courses of the tyrosine phosphorylation of both the receptor and the kinase are compatible with previous data that JAK2 preassociates with the receptor and becomes activated after the PRLR-ligand binding presumably by transphosphorylation of tyrosines. The phosphorylated tyrosine on PRLR binds with the Src homology 2 domain of a STAT to recruit it, and the JAK 2 phosphorylation of the receptor-bound STAT causes the release of STAT and its translocation into the nucleus, where the STATs become transcriptionally active (32). In agreement with this mode of interaction, the increase in tyrosine phosphorylation of STATs occurred with a time course compatible with it being a downstream event for prolactin-dependent JAK2 activation in PC12 cells. In PC12 cells prolactin mediated tyrosine phosphorylation of Stat1, -3, and -5a, with similar time courses and regulation. While all three are considered as important effectors for PRLR, genetic analysis has indicated that Stat5a is the most important in the prolactin-specific biological effect of lactogenesis (17). However, since the question has not yet been
directly addressed, it remains unclear whether Stat5a is important in the temporally prerequisite alveolar cell proliferation or the following alveolar cell differentiation or both. In most studies, Stat5a activation usually correlates with differentiation, including terminal differentiation of mammary secretory epithelium cells (33). This is in agreement with the gain in morphological differentiation we observed in PC12 cells.

**Prolactin Activates Raf-B but Not ERK 1/2 in PC12 Cells—** Prolactin acutely induced increases in the phosphorylation of Raf-B (Fig. 3). Prolactin induces rapid phosphorylation and activation of Raf-1 (Raf-C) kinase in the Nb2 T-cell line, where the PRL receptor also co-immunoprecipitates with Raf-1 (18). Most receptors of the cytokine class-1 family activate Raf-1 by tyrosine phosphorylation. In contrast, PRLR activates Raf-1 with a mechanism similar to that of JAK2 activation; i.e. agonist binding stimulates autokinase activity. This fundamental difference between PRLR and the other receptors has been proposed as point of specificity for the prolactin signaling (18). The time course of the PRL-induced activation is compatible with a similar association of Raf-B with PRLR in PC12 cells.

One surprising finding was that prolactin activated Raf-B, but not its classic downstream effector in mitogenic pathways, the MAP kinase ERK1 or -2 (for PC12 cells (34)). However, the coupling of prolactin to ERK activation appears to be cell type-dependent (35, 36), while the recent finding that the coupling of PRL to ERK may require a soluble factor secreted only under specific cell density (19) suggests that the coupling may be additionally dependent on stages of differentiation within a cell lineage. Moreover, in epithelial cells prolactin may act as an inhibitor of the ERK pathway activated by fibroblast growth factor, vascular endothelial growth factor (37), or EGF (38). The coupling of PRL to the ERKs has not been previously addressed in neuronal cell types. Prolactin activates ERK1 and -2 in postmitotic cortical neurons and in astrocytes (4). We were, however, unable to demonstrate ERK activation by immunoblotting with ERK antibodies (Fig. 3C), antibodies against activated ERK, immunocytochemistry, or myelin basic protein phosphorylation assays. In sister cultures, processed simultaneously, we showed that, as expected (30), EGF transiently activated ERK1 and -2 in PC12 cells (Fig. 3C). Therefore, the possibility that an ERK activation by PRL was overlooked is unlikely.

Prolactin, however, activated the MAP kinase JNK and subsequently c-Jun expression in PC12 cells. c-Jun is a member of the bZip family of transcription factors (39), which can combine with c-Fos to form homo- and heterodimers, with Jun-Jun homodimers, or Jun-Fos heterodimers (referred to as the AP-1 complex), typically to activate gene transcription. Increases in Jun were observed both with immunodetection (Fig. 4) and reverse transcription-PCR. PC12 cells were treated with prolactin for 30 min, and the reverse transcription-PCR-generated cDNA of c-Jun was used to generate the GST-c-Jun fusion protein used in the JNK assays. c-Jun cDNA could not be amplified from equivalent amounts of total RNA, derived from control PC12 cells, providing additional evidence that prolactin stimulated c-Jun expression. The increase in c-Jun phosphorylation was evident within 15 min of incubation with prolactin and was accompanied by an increase in c-Jun levels, which lasted for hours (Fig. 4). Activation of c-Jun has been considered a negative regulator of the human or rat prolactin promoter, however, in the context of forming AP-1 complexes (40). Specific prolactin-dependent c-Jun activation has been reported in metastases of pituitary tumors (41).

Moreover, using solid phase JNK activity assays and GST-c-Jun(1–79) fusion protein as a substrate for JNK, we measured a sustained activation of JNK activity. JNKs, a group of serine-threonine kinases structurally related to the ERKs, are activated by tyrosine kinases, during processes where drastic changes in membrane shape occur downstream to Ras/Raf activation. JNK activation has been also associated with induction of apoptosis in PC12 cells (42). Our time lapse photography, together with increased rates of both growth and BrdUrd uptake, showed that prolactin did not induce apoptosis in PC12 cells. PRL, at concentrations that usually stimulate cell proliferation, can protect cells against glucocorticoid receptor-mediated apoptosis. Moreover, JNK is mostly associated with mitotic events rather than with apoptosis (43). Thus, JNK is activated in response to EGF and the phorbol esters in most cell types that the coupling has been investigated, albeit with a shorter time course of activation (44). Hashimoto et al. (45) have suggested that EGF-mediated ERK activation depends on Grb2, whereas EGF-mediated JNK activation is dependent on Shc. Similar disengagement of ERK from a proliferation signaling cascade and concurrent detection of increased JNK activity was recently reported in cells growing in the presence of the cytokine IL-4 (35). JNK activity was increased by IL-4 only if cells expressed an activated mutant of Raf-1. Taken together, the view of activation of Raf/ERK as distinct from JNK activation may need to be reassessed, since activation of RAF may influence JNK activity.

The possibility that activation of JNK may also reflect differentiation-related events cannot be excluded with our studies. However, our studies at the single cell level clearly postulate a role for JNK in PRL-induced mitosis (Fig. 5). Low concentration of SB203580, inhibition of p38 or PD98059, and inhibition of MEK and ERKs had no effect on PRL-induced BrdUrd incorporation, whereas 30 μM SB203580 and JNK inhibition abolished it. In contrast, EGF-induced BrdUrd incorporation, independent of SB203580 and p38 or JNK activation, was mediated by ERK activation. Similar experiments with primary astrocytes, where prolactin concurrently mediates mitosis and up-regulation of astrocytic differentiation-specific genes showed that BrdUrd incorporation was inhibited with 30 μM SB203580.2 Last, no significant increases of serum or threonine phosphorylation on immunoprecipitated STATs were observed in response to PRL, and therefore the possibility that JNK may mediate its effects after serine phosphorylation of the STATs as reported for EGF (47) was also excluded.

In conclusion, our studies showed that prolactin activates a mitogenic signaling pathway with a concurrent gain in differentiation. Our data agree with the general notion that prolactin is a growth factor that accesses most cell types as a pituitary hormone or a locally secreted factor and identify JNK activation as a major event in the PRL mitogenic signaling in PC12 cells.

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