Prolactin-releasing Peptide Activation of the Prolactin Promoter Is Differentially Mediated by Extracellular Signal-regulated Protein Kinase and c-Jun N-terminal Protein Kinase*

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Regulation of the mitogen-activated protein kinase (MAPK) family by prolactin-releasing peptide (PrRP) in both GH3 rat pituitary tumor cells and primary cultures of rat anterior pituitary cells was investigated. PrRP rapidly and transiently activated extracellular signal-regulated protein kinase (ERK) in both types of cells. Both pertussis toxin, which inactivates G<sub>i</sub>/G<sub>o</sub> proteins, and exogenous expression of a peptide derived from the carboxyl terminus of the β-adrenergic receptor kinase I, which specifically blocks signaling mediated by the βγ subunits of G proteins, completely blocked the PrRP-induced ERK activation, suggesting the involvement of G<sub>i</sub>/G<sub>o</sub> proteins in the PrRP-induced ERK activation. Down-regulation of cellular protein kinase C did not significantly inhibit the PrRP-induced ERK activation, suggesting that a protein kinase C-independent pathway is mainly involved. PrRP-induced ERK activation was not dependent on either extracellular Ca<sup>2+</sup> or intracellular Ca<sup>2+</sup>. However, the ERK cascade was not the only route by which PrRP communicated with the nucleus. JNK was also shown to be significantly activated in response to PrRP. JNK activation in response to PrRP was slower than ERK activation. Moreover, to determine whether a MAPK family cascade regulates rat prolactin (rPRL) promoter activity, we transfected the intact rPRL promoter ligated to the firefly luciferase reporter gene into GH3 cells. PrRP activated the rPRL promoter activity in a time-dependent manner. Co-transfection with a catalytically inactive form of a MAPK construct or a dominant negative JNK, partially but significantly inhibited the induction of the rPRL promoter by PrRP. Furthermore, co-transfection with a dominant negative Ets completely abolished the response of the rPRL promoter to PrRP. These results suggest that PrRP differentially activates ERK and JNK, and both cascades are necessary to elicit rPRL promoter activity in an Ets-dependent mechanism.

Prolactin (PRL) is important in pregnancy and lactation in mammals, and is involved in the development of the mammary glands and the promotion of milk synthesis (1). Thyrotropin-releasing hormone (TRH) is a physiological regulator of pituitary cell function that stimulates prolactin synthesis and secretion (2). Recently, a new peptide which is a ligand of the “orphan” receptor hGR3 expressed specifically in the hypothalamus was identified in the hypothalamus as a potent prolactin-releasing factor for rat anterior pituitary cells (3). This peptide was named “prolactin-releasing peptide” (PrRP).

The receptor of PrRP, hGR3, is referred to as a seven-transmembrane domain receptor or a G protein-coupled receptor (4, 5). Although it was reported that PrRP induced arachidonic acid metabolite release as well as PRL secretion (3), the signal transduction pathway in PrRP-induced PRL secretion or synthesis has remained unknown. The effects of TRH are presumably mediated by activation of phosphatidylinositol 4,5-bisphosphate-phospholipase C, leading to the production of inositol phosphates and diacylglycerol (6, 7). Indeed, many of the downstream effects of TRH are believed to be dependent on mobilization of intracellular calcium and activation of protein kinase C (PKC). Although G protein-coupled receptors are thought to be linked primarily to second messenger systems, protein tyrosine phosphorylation can occur soon after receptor occupancy in some cases (8, 9).

Intracellular transmission of extracellular signals is mediated in large part by several groups of sequentially activated protein kinases, which are collectively known as the mitogen-activated protein kinase (MAPK) cascade. In growth factor signaling, the key elucidated MAPK cascade is that involving extracellular signal-regulated kinase (ERK). Recent evidence indicates that some G protein-coupled receptors can activate the ERK cascade (10–12). The signals transmitted through the ERK cascade lead to activation of a set of regulatory molecules that ultimately initiate cellular responses such as growth and differentiation (13–15). Recently we have shown that TRH is capable of activating ERK in pituitary organ culture (16) and in GH3 rat pituitary tumor cells (10), and that ERK might be involved in PRL secretion or synthesis (17).
However, the ERK cascade is not the only link between membrane receptors and their intracellular targets, and several other ERK-like cascades have been identified (18). One of the most studied of these cascades is the Jun N-terminal kinase (JNK; also known as stress-activated protein kinase (SAPK) (19, 20)) cascade, which is activated in response to cellular stresses such as apoptosis (19, 21), ERK, JNK, and p38 (22) are members of the MAPK family. Recent data indicate that GnrRH is capable of activating ERK (23, 24), JNK (25), and p38 (26) in the αT3-1 gonadotroph cell line.

It has been shown that Raf, ERK, and JNKs are crucial components of the downstream transmission of the Ras signal in the regulation of the PRL promoter activity (27, 28). The JNK family of transcription factors, which comprises a number of phosphoproteins with a conserved DNA-binding motif named the JNK domain (29), have been demonstrated to be phosphorylated and activated by ERK (30). Several JNK-binding sites have been identified in the proximal PRL promoter.

Taken together, these facts led us to examine whether PrRP stimulates the activity of ERK or JNK, and whether each of these cascades plays a role in the transcriptional activation of the rat PRL (rPRL) gene in GH3 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phorbol 12-myristate 13-acetate (PMA) and myelin basic protein were purchased from Sigma. PrRP was a gift from Takeda Chemical Industries Ltd. (Japan). ECL Western blotting detection reagents were obtained from Amersham Pharmacia Biotech. 1, γ-32P]ATP (3000 Ci/mmol) was obtained from NEN Life Science Products Inc. Erk1 rabbit polyclonal anti-ERK antiserum and monoclonal antibody 9E10 to the NH2-terminal: this site contains two phosphorylation sites at Ser63 and Ser73. The beads were washed and resuspended in 50 μl of kinase buffer containing 100 μM MgCl2, 10 mM MnCl2, and 1 mM dithiothreitol, and 300 μg of protein from the lysate samples was immunoprecipitated at 4 °C for 2 h. The immunoprecipitated proteins were resolved on 7.5% T.L LiCl. 0.1 M Tris, pH 8.0, and once in kinase assay buffer (25 mM HEPES, pH 7.2–7.4, 10 mM MgCl2, 10 mM MnCl2, and 1 mM dithiothreitol), and samples were resuspended in 30 μl of kinase assay buffer containing 10 μg of myelin basic protein and 40 μM 1, γ-32P]ATP (1 μCi) as described previously (23). The kinase reaction was allowed to proceed at room temperature for 5 min and stopped by the addition of Laemmli SDS sample buffer (43). Reaction products were resolved by 15% SDS-PAGE.

**Assay of 49-kDa ERK Activity Using a Transient Expression System**—GH3 cells cultured in 100-mm dishes were transfected with Myc-tagged p42MAPK expression plasmid (1 μg of pEXV-Erk2-tag) in combination with 9 μg of pPRK or pPRK-JAK1 using LipofectAMINE as described previously (12, 44). At 72 h after transfection, serum-deprived cells were incubated with 1 μg PrRP for 5 min, and expressed Myc-tagged p42MAPK was immunoprecipitated with 1 μg of anti-Myc antibody (Ser61). The ERK activity in the immunoprecipitate was measured as described above. The transfection efficiency of each experiment was 8–10% as assessed by β-galactosidase staining after transfection of a β-galactosidase-containing expression plasmid.

**Assay of JNK Activity**—JNK activity was precipitated from 250 μg of whole cell lysates by incubation with 2 μg of GST-c-Jun (1–89) fusion protein/GSH-Sepharose beads for 18 h at 4 °C (45). The kinase reaction was allowed to proceed at room temperature for 5 min. In some of the experiments, serum-deprived cells were incubated with 10 μM PrRP for 15 min before the addition of GST-JNK to the homogenate. JNK cell extracts were prepared by lysing the cells with three sequential freeze-thaw cycles in a buffer containing 100 mM potassium phosphate, pH 7.8, and 10 mM dithiothreitol. Vigorous vortexing was used to enhance cell lysis. Unlysed cells and insoluble material were pelleted at 10,000 rpm for 10 min at 4 °C. The supernatant volume was measured, and aliquots of the supernatant were used in the subsequent luciferase and β-galactosidase assays.

Luciferase was assayed as described previously (34). Briefly, the luciferase assay mixture contained 100 mM KPO4, pH 7.8, 1 mM dithiothreitol, 3.7 mM MgSO4, 530 μM ATP, and 470 μM luciferin plus 20 μl of cell extract in a final volume of 100 μl. Luciferin was added just before measuring light units, which were measured in duplicate during the first 40 s of the reaction at 25 °C in a Lumatometer (Dinamitek, Tustin, CA). β-Galactosidase was assayed as described previously (34). The β-galactosidase buffer contained 60 mM sodium phosphate, pH 7.5, 1 mM MgCl2, 0.80 mg/ml o-nitrophenyl-β-d-galactoside, and 40 μM β-mercaptoethanol. A standard curve containing 100 micromolar concentrations of β-galactosidase was made with each assay. A 30-μl aliquot of cell extract was incubated with assay buffer until color developed (30–120 min), and the reaction was then stopped by adding Na2CO3 to a final concentration of 0.3%. Absorbance was measured at 405 nm.

Luciferase light units were normalized relative to the activity of β-galactosidase. The control value was then set at 1 and the data were expressed as fold-stimulation relative to control. Data are expressed as the mean ± S.E.

**Statistics**—Statistical analysis was performed by Student’s t test, and p < 0.01 was considered significant. Data are expressed as the mean ± S.E.
**RESULTS**

**PrRP Stimulation of ERK Activity**—Recently we reported that the activity of ERK is stimulated by TRH in GH3 cells (10). TRH is a potent factor that is known to be capable of promoting both PRL secretion and synthesis (2). PrRP was comparable to TRH in its potency (3). PrRP acts through a specific receptor, which is referred to as a seven-transmembrane domain receptor or G protein-coupled receptor (4, 5). We therefore investigated whether PrRP might induce the activation of ERK. GH3 cells were treated with 1 μM PrRP for the indicated times. Activation of ERK was evaluated (Fig. 1A, left panel). Cells were treated with 1 μM PrRP for 5 min (lane 2) or 3 h (lane 3). B, cells were treated with the indicated concentrations of PrRP for 5 min. Lysates of cells were subsequently immunoprecipitated (I.P.) with anti-ERK antiserum, and the immunoprecipitates were incubated with [γ-32P]ATP in the presence of MBP, as described under “Experimental Procedures.” After the reactions were stopped with Laemmli sample buffer, SDS-PAGE and autoradiography were performed. Autoradiograms of 32P-labeled MBP are shown in the upper panel, with the density of the control bands set arbitrarily at 1.0. Values shown represent the mean ± S.E. from at least three separate experiments. **a** indicates p < 0.01 as compared with the control.

**Gβγ-mediated PrRP-induced ERK Activation**—It has been shown that the receptors for both TRH (6, 7) and PrRP (4, 5) are members of the superfamily of G protein-coupled receptors. We compared the mechanisms of ERK activation induced by each TRH and PrRP. To determine what type of G protein is coupled to each receptor, we pretreated GH3 cells (Fig. 2A, left panel) or primary cultures of rat anterior pituitary cells (Fig. 2A, right panel) with 100 ng/ml pertussis toxin (PTX) for 4 h in order to inactivate Gα and Gβγ proteins, and then treated the cells with 1 μM PrRP or TRH for 5 min. Although PTX at 100 ng/ml almost completely blocked the PrRP-induced ERK activation (Fig. 2A, lane 4), PTX did not have an apparent effect on TRH-induced ERK activation (Fig. 2A, lane 6) in both types of cells. Thus, the effect of PrRP on ERK activity involves PTX-sensitive G proteins such as Gαi or Gαo, whereas that of TRH does not involve PTX-sensitive G proteins.

It has been reported that the carboxyl terminus of the β-adrenergic receptor kinase, containing its Gβγ-binding domain, is a cellular Gβγ antagonist capable of specifically distinguishing Gαi- and Gβγ-mediated processes (33). To examine the effect of the Gβγ subunit-sequestrant βARKct peptide on PrRP-induced exogenous ERK activity, a Myc-tagged p42mapk expression plasmid (pEXV-ERK2-tag) and, after 72 h, were stimulated with 1 μM PrRP (lanes 2 and 4). Autoradiograms of ERK activity immunoprecipitated with antibody to the Myc epitope and assayed by 32P incorporation into MBP are shown in the lower panel. Relative densitometric units of the MBP bands is similar to those which stimulate the release of PRL (3).

**Role of PKC in Activation of ERK**—Many G protein-linked receptors can mediate stimulation of ERK activity via the phospholipase C-dependent activation of PKC (47, 48). Activation of ERK by TRH requires PKC in GH3 cells (10). The role of PKC in PrRP-induced ERK activation was examined (Fig. 3). Exposure of GH3 cells to PMA caused a stimulation of ERK by TRH requires PKC in GH3 cells (10). Therefore, the role of PKC in PrRP-induced ERK activation was examined (Fig. 3). Exposure of GH3 cells to PMA caused a stimulation of ERK by TRH requires PKC in GH3 cells (10). Therefore, the role of PKC in PrRP-induced ERK activation was examined (Fig. 3). Exposure of GH3 cells to PMA caused a stimulation of ERK by TRH requires PKC in GH3 cells (10). Therefore, the role of PKC in PrRP-induced ERK activation was examined (Fig. 3). Exposure of GH3 cells to PMA caused a stimulation of ERK by TRH requires PKC in GH3 cells (10). Therefore, the role of PKC in PrRP-induced ERK activation was examined (Fig. 3). Exposure of GH3 cells to PMA caused a stimulation of ERK by TRH requires PKC in GH3 cells (10). Therefore, the role of PKC in PrRP-induced ERK activation was examined (Fig. 3). Exposure of GH3 cells to PMA caused a stimulation of ERK by TRH requires PKC in GH3 cells (10). Therefore, the role of PKC in PrRP-induced ERK activation was examined (Fig. 3). Exposure of GH3 cells to PMA caused a stimulation of ERK by TRH requires PKC in GH3 cells (10). Therefore, the role of PKC in PrRP-induced ERK activation was examined (Fig. 3). Exposure of GH3 cells to PMA caused a stimulation of ERK by TRH requires PKC in GH3 cells (10). Therefore, the role of PKC in PrRP-induced ERK activation was examined (Fig. 3). Exposure of GH3 cells to PMA caused a stimulation of ERK by TRH requires PKC in GH3 cells (10). Therefore, the role of PKC in PrRP-induced ERK activation was examined (Fig. 3). Exposure of GH3 cells to PMA caused a stimulation of ERK by TRH requires PKC in GH3 cells (10). Therefore, the role of PKC in PrRP-induced ERK activation was examined (Fig. 3).
as in the case of norepinephrine-induced ERK activation in both adipocytes (49) and GT-1 GnRH neuronal cell lines (50). Whether PKC is indeed involved in PrRP signaling was determined using PKC depletion. Pretreatment with 1 μM PMA for 16 h to deplete most PKC isoforms partially attenuated the PrRP- (Fig. 3, lane 3) and completely abolished the TRH- (Fig. 3, lane 5) induced ERK activation. These results suggest that activation of ERK by TRH is mainly mediated by PKC and activation of ERK by PrRP is partly mediated by PKC.

Role of Extracellular and Intracellular Ca2+ in ERK Activation—It has been reported that Ca2+ influx is important as a signal-transduction pathway in PRL secretion by pituitary cells (51) and that PrRP could induce Ca2+ influx (3). We therefore evaluated the effect of Ca2+ influx on the PrRP- and TRH-induced ERK activation (Fig. 4A). Elimination of extracellular Ca2+ by treatment with 3 mM EGTA for 1 min completely blocked the TRH-induced ERK activation (Fig. 4A, lane 7), indicating that Ca2+ influx is required for TRH-induced ERK activation. Interestingly, neither elimination of extracellular calcium by treatment with 3 mM EGTA for 1 min nor elimination of both extracellular and intracellular Ca2+ by treatment with 3 mM EGTA for 15 min (52) attenuated the PrRP-induced ERK activation (Fig. 4A, lanes 3 and 4). Moreover, treatment with 50 μM BAPTA-AM for 20 min to eliminate intracellular Ca2+ had no effect on PrRP-induced ERK activation (Fig. 4A, lane 5). Next, the effect of extracellular and intracellular Ca2+ on PrRP-induced ERK activation was examined in primary cultures of rat anterior pituitary cells (Fig. 4B). Incubation in calcium-free medium, elimination of extracellular Ca2+ by treatment with 3 mM EGTA for 1 min, and elimination of intracellular Ca2+ by treatment with 50 μM BAPTA-AM for 20 min had no effect on PrRP-induced ERK activation. These results suggest that TRH-induced ERK activation is completely dependent on extracellular Ca2+, whereas PrRP-induced ERK activation is dependent on neither extracellular Ca2+ nor intracellular Ca2+.

Stimulation of JNK Activity by PrRP and TRH—To determine whether JNK activity was affected by PrRP or TRH, we used a GST-cJun (1–89) fusion protein bound to GSH-Sepharose beads to precipitate the JNK activity from GH3 cell lysates. The precipitated complex was subjected to an in vitro solid-phase kinase assay, and then phosphorylation on Ser63 was measured by Western blotting with anti-phospho(Ser63) c-Jun antibody. JNK activity was clearly stimulated by both PrRP (Fig. 5A, left panel) and TRH (Fig. 5A, right panel). JNK activation was detected 5 min after the initiation of the PrRP or TRH treatment, it peaked by 3 h, and decreased over the next 16 h. Thus, JNK activation by PrRP was slower than its ERK activation (Fig. 1A). Next, we examined the effect of PTX on the PrRP-induced JNK activation. Pretreatment with 100 ng/ml PTX for 4 h did not completely inhibit the PrRP-induced JNK activation (Fig. 5B, lane 3), which was different from the effect of PTX on the PrRP-induced ERK activation (Fig. 2A). In addition, the role of PKC in the PrRP-induced JNK activation was examined. Pretreatment with 1 μM PMA for 16 h significantly inhibited the PrRP-induced JNK activation (Fig. 5B, lane 5) as was also true for the PrRP-induced ERK activation (Fig. 3, lane 6).
An Ets Transcription Factor Is a Nuclear Acceptor of the MAPK Family Signaling Cascade—Members of the recently characterized Ets transcription factor family contain a transactivation domain at the amino terminus and a highly conserved DNA-binding domain at the carboxyl terminus, and this latter domain defines the Ets family of transcription factors since it lacks homology to other DNA-binding motifs (55). Members of the ternary complex factor subfamily of Ets transcription factors are also targets of MAP kinase cascades (56). The Ets-domain transcription factor Elk-1 is a substrate for three distinct classes of MAP kinase family members (56–58). In addition, previous studies from other laboratories have suggested that Ets transcription factors mediate the response of certain growth factors—such as transforming growth factor-1 (61), and fibroblast growth factor (62)—to the involvement of the JNK cascade in the stimulation of the rPRL promoter by PrRP. An expression plasmid that encodes a dominant negative SAPK/JNK (pcDL-SRα-SAPK-VPF) was used to inhibit the JNK cascade (41). Co-transfection with pcDL-SRα-SAPK-VPF significantly attenuated the PrRP-induced rPRL promoter activation (Fig. 6B), suggesting that the JNK cascade is also involved in the PrRP-induced rPRL promoter activation.

An Ets Transcription Factor Is a Nuclear Acceptor of the MAPK Family Signaling Cascade—Members of the recently characterized Ets transcription factor family contain a transactivation domain at the amino terminus and a highly conserved DNA-binding domain at the carboxyl terminus, and this latter domain defines the Ets family of transcription factors since it lacks homology to other DNA-binding motifs (55). Members of the ternary complex factor subfamily of Ets transcription factors are also targets of MAP kinase cascades (56). The Ets-domain transcription factor Elk-1 is a substrate for three distinct classes of MAP kinase family members (56–58). In addition, previous studies from other laboratories have suggested that Ets transcription factors mediate the response of certain growth factors—such as transforming growth factor-1 (61), and fibroblast growth factor (62). Therefore, these findings led us to examine whether Ets transcription factors are the nuclear acceptors for PrRP signaling. To examine the functional role of Ets transcription factors in PrRP-induced activation of the rPRL promoter, we next examined the possibility of the involvement of the JNK cascade in the stimulation of the rPRL promoter by PrRP. An expression plasmid that encodes a dominant negative SAPK/JNK (pcDL-SRα-SAPK-VPF) was used to inhibit the JNK cascade (41). Co-transfection with pcDL-SRα-SAPK-VPF significantly attenuated the PrRP-induced rPRL promoter activation (Fig. 6B), suggesting that the JNK cascade is also involved in the PrRP-induced rPRL promoter activation.

**Fig. 6.** Stimulation of the rPRL promoter activity by PrRP through ERK and JNK cascades. A, GH3 cells were transiently co-transfected with 0.4 μg of the reporter construct pA3–425PRLluc and 0.04 μg of an internal control, pCMVβgal. After transfection, cells were treated with 1 μM PrRP for the indicated times prior to harvesting. B, GH3 cells were transiently co-transfected with 0.4 μg of the reporter construct pA3–425PRLluc and 0.04 μg of an internal control, pCMVβgal, with or without 1.2 μg of pAPr or pAPr-etsZ (A) or 1.2 μg of pRK5, pRK5-ets-2Δ1–328, or pRK5-ets-2 (B), as indicated. After transfection, cells were treated with 1 μM PrRP for 12 h prior to harvesting. Luciferase activity was normalized relative to β-galactosidase activity, and the basal activity of pA3–425PRLluc was set at 1.0. Data are expressed as the mean fold activation ± S.E. of six transfections. ** indicates p < 0.01 as compared with the respective control.

**Fig. 7.** Dominant-negative Ets inhibits PrRP activation of the rPRL promoter. GH3 cells were transiently co-transfected with 0.4 μg of the reporter construct pA3–425PRLluc and 0.04 μg of an internal control, pCMVβgal, with or without 1.2 μg of pAPr or pAPr-etsZ (A) or 1.2 μg of pRK5, pRK5-ets-2Δ1–328, or pRK5-ets-2 (B), as indicated. After transfection, cells were treated with 1 μM PrRP for 12 h prior to harvesting. Luciferase activity was normalized relative to β-galactosidase activity, and the basal activity of pA3–425PRLluc was set at 1.0. Data are expressed as the mean fold activation ± S.E. of six transfections. ** indicates p < 0.01 as compared with the respective control.
induced rPRL promoter activation (Fig. 7B). Thus, the inhibitory effect of pRK5-ets-2Δ1-328 on the PrRP-induced rPRL promoter activation appeared to be due to interference with activated Ets-2. These results suggest that a member of the Ets transcription factor family is a nuclear acceptor for the stimulation of rPRL promoter activity by PrRP.

**DISCUSSION**

PrRP was isolated as the ligand of an “orphan receptor,” which is a seven-transmembrane domain receptor specifically expressed in the pituitary (3). PrRP induced arachidonic acid metabolite release as well as PRL secretion in both primary cultured rat anterior pituitary cells and a rat pituitary adenoma-derived cell line, RC-4B/C (3). Since TRH is a potent factor capable of promoting both PRL secretion and synthesis (2), we considered the possibility that PrRP can also act on PRL synthesis as well as PRL release. We reported previously that TRH rapidly and transiently induced ERK activation (10, 16). PrRP was almost as potent as TRH in the ability to induce ERK activation in both GH3 cells and primary cultured rat anterior pituitary cells (Fig. 2A). It is well known that either extracellular Ca$^{2+}$ or intracellular Ca$^{2+}$ is involved in the induction of PRL secretion by TRH (51). Although PrRP could induce Ca$^{2+}$ influx in CHO-19P2 cells (3), PrRP-induced ERK activation was not dependent on either extracellular or intracellular Ca$^{2+}$ in GH3 cells (Fig. 4A) or in primary pituitary cultures (Fig. 4B). In addition, the time frame of PrRP-induced ERK activation (Fig. 1A) was not as rapid as that of PrRP-induced intracellular Ca$^{2+}$ mobilization (data not shown). These facts led us to examine the effect of PrRP on PRL synthesis and the role of the ERK cascade in PRL synthesis. PrRP activated rPRL promoter activity in a time-dependent fashion (Fig. 6A). Either pretreatment with PD98059 or co-transfection with an iMAPK-encoding construct to block the ERK cascade significantly inhibited PrRP-induced rPRL promoter activation. These data suggest that the ERK cascade might be involved in the PrRP-induced PRL synthesis.

Distinct pathways of G$_i$- and G$_q$-mediated ERK activation have been reported (33). The activation of G$_i$-coupled receptors, such as thrombin (69), oxytocin (11), prostaglandin F$_2$α (12), and endothelin-1 (44), appears to be PTX-sensitive and PKC-independent. In addition, G$_q$-mediated ERK activation is initiated by phosphatidylinositol 3-kinase activity, followed by a pathway common to tyrosine kinase receptors (64). However, the case of receptors that couple to G$_q$, such as bombesin, activation is thought to be secondary to stimulation of phosphatidylinositol 4,5-bisphosphate-phospholipase C, leading to production of inositol phosphate and diacylglycerol, with subsequent PKC-mediated stimulation of ERK (47). TRH binds to a G protein-coupled receptor, presumably of the PTX-insensitive G$_q$ family, and activates multiple signaling pathways in pituitary cells (10). In this study, pretreatment with PTX did not apparently block the TRH-induced ERK activation (Fig. 2A) and apparent down-regulation of PKC by prolonged incubation with PMA attenuated the stimulation of ERK activity by TRH (Fig. 3), confirming the involvement of PTX-insensitive G$_q$-protein kinase C in TRH-induced ERK activation. On the other hand, both pretreatment with PTX and expression of βARK1 blocked the PrRP-induced ERK activation (Fig. 2) and down-regulation of PKC by prolonged incubation with PMA did not apparently attenuate the stimulation of ERK activity by PrRP (Fig. 3), suggesting that PrRP stimulation of ERK activity is not likely to be mainly mediated by G$_q$-protein kinase C, but to be mediated by a PTX-sensitive G protein (G$_q$ or G$_i$).

Ca$^{2+}$ is a critical mediator of the induction of PRL secretion by TRH in both primary cultures of rat anterior pituitary cells and primary pituitary cultures (Fig. 4A). In addition, the regulation of the PRL promoter by TRH is dependent on Ca$^{2+}$ influx (66). Elimination of extracellular Ca$^{2+}$ by treatment with 3 mM EGTA for 1 min completely abolished the TRH-induced ERK activation (Fig. 4A). On the other hand, elimination of extracellular Ca$^{2+}$ by treatment with 3 mM EGTA for 1 min (Fig. 4), extracellular Ca$^{2+}$ and intracellular Ca$^{2+}$ by treatment with 3 mM EGTA for 15 min (Fig. 4A), or intracellular Ca$^{2+}$ by treatment with 50 μM BAPTA-AM for 20 min (Fig. 4) did not attenuate PrRP-induced ERK activation. These results also confirmed that the mechanism of PrRP-induced ERK activation might be different from that of TRH-induced ERK activation.

One important downstream biochemical event that occurs after ligand binding to many growth-promoting receptors is the activation of members of the MAP kinase family, including ERK and JNK (22). The existence of parallel cascades leading to activation of either ERK or JNK was reported. PrRP induced the activation of both ERK and JNK. Is the mechanism of PrRP-induced ERK activation different from that of PrRP-induced JNK activation? PrRP activated ERK in a partly PKC-dependent, extracellular and intracellular Ca$^{2+}$-independent manner (Figs. 3 and 4). Since EGTA itself induced JNK activation in GH3 cells (data not shown), the effect of EGTA on PrRP-induced JNK activation could not be examined. PrRP activated JNK in a PKC-dependent manner (Fig. 5B). Interestingly, although PTX completely inhibited the PrRP-induced ERK activation (Fig. 2), PTX only partially inhibited the PrRP-induced JNK activation (Fig. 5B). Moreover, the time course of the JNK activation (Fig. 5A) in response to PrRP was slower than that of ERK activation (Fig. 1A). Thus, the regulation of the JNK activation by PrRP appeared to be different from that of the ERK activation.

PrRP-induced activation of the rPRL promoter was attenuated by either pretreatment with MEK inhibitor PD98059 or co-transfection with an iMAPK construct (Fig. 6B), suggesting the requirement of the ERK cascade for the PrRP-induced rPRL promoter activation. Since PrRP-induced transcription of the rPRL gene was not fully blocked by either pretreatment with PD98059 or co-transfection with an iMAPK construct, it is likely that intracellular cascades other than the ERK cascade are also involved in transducing the transcriptional effects of PrRP. Since JNK activity was also stimulated by PrRP (Fig. 5), there is a possibility that the JNK cascade is also involved in the PrRP-induced rPRL promoter activation. PrRP-induced activation of the rPRL promoter was attenuated by co-transfection with a dominant negative SAPK/JNK construct (Fig. 6B), suggesting the requirement of the JNK cascade for the PrRP-induced rPRL promoter activation.

Transcription factors binding to a PrRP-responsive region of the rPRL promoter have not been identified. ERKs have been reported to phosphorylate the ternary complex factor Elk-1, which controls the expression of the c-fos gene (67, 68). It has been demonstrated that JNK phosphorylates c-Jun and ATF-2 at the putative regulatory amino-terminal serine residues and increases their transcriptional activities (19, 20, 69). Moreover, JNK has been reported to activate Elk-1, resulting in an increase in c-fos gene expression (70). However, the proximal rPRL promoter does not contain ATF/CREB sites. Although the proximal rPRL promoter does not contain any consensus AP-1 sites (TGA/C/G/TCA) (71), it is conceivable that c-Jun might still be involved as a nuclear acceptor of a JNK signal. Therefore, we used dnJun to examine whether c-Jun might be involved as a nuclear acceptor of the JNK signal. DnJun has been characterized and successfully used for the derivative acts at a point distal to JNK in the JNK signal transduction cascade in a number of studies (72, 73). Co-transfection of a dnJun expres-
sion vector had no effect on the PrRP-induced rPRL promoter activity (data not shown), suggesting that c-Jun is not a substrate for JNK in the PrRP-induced rPRL promoter activation. By contrast, several putative Ets sites (5′/ACGGAA), located at positions −295, −185, and −165, are found in the rPRL promoter. Ets, which appears to mediate transcriptional responses to the ERK cascade, is an important component in the regulation of lactotroph-specific SAPK gene expression (74) and in the regulation of the rPRL promoter in response to Ras (27, 28), insulin (59, 60), insulin-like growth factor-1 (61), and fibroblast growth factor (62). These data suggest that activation of the ERK cascade leading to the phosphorylation of an Ets factor could be involved in the activation of the rPRL promoter by PrRP. Co-transfection with either pAPr-ets-Z (Fig. 7A) or an pRK5-ets-Δ21–328 (Fig. 7B) completely inhibited the PrRP-induced rPRL promoter activation. Moreover, the Ets-domain transcription factor Elk-1 is a substrate for both ERK and JNK (56). Thus, there is a possibility that PrRP might use both ERK and JNK cascades to elic rPRL promoter activity with the Ets sites as the responsible region.

The signaling cascades that couple the activation of PrRP receptor to transcription are not yet fully defined. Since Gα-mediated ERK activation is initiated by phoshoatidylinositol 3-kinase activity (64) and wortmannin, an inhibitor of phosphatidylinositol 3-kinase, prevents the response of the rPRL promoter to insulin-like growth factor-1 (61), potential candidates for such cascades include those mediated by phoshoatidylinositol 3-kinase. In addition, it remains to be determined whether other MAP kinase family members such as p38 or the newly described SAPK3 (22), are also activated by PrRP. Moreover, the complete role of the MAP kinase family in the action of PrRP in lactotrophs remains to be explored. Apart from a contribution to mediating transcriptional responses to PrRP, either ERK or JNK activation may be associated with other yet unknown cellular responses to PrRP, such as effects on long-term maintenance of the lactotroph phenotype.

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Prolactin-releasing Peptide Activation of the Prolactin Promoter Is Differentially Mediated by Extracellular Signal-regulated Protein Kinase and c-Jun N-terminal Protein Kinase

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