Divergent Signaling Pathways Requiring Discrete Calcium Signals Mediate Concurrent Activation of Two Mitogen-activated Protein Kinases by Gonadotropin-releasing Hormone*

Received for publication, November 30, 1999, and in revised form, March 2, 2000

Jennifer M. Mulvaney and Mark S. Roberson‡

From the Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

Receptors coupled to heterotrimeric G proteins are linked to activation of mitogen-activated protein kinases (MAPKs) via receptor- and cell-specific mechanisms. We have demonstrated recently that gonadotropin-releasing hormone (GnRH) receptor occupancy results in activation of extracellular signal-regulated kinase (ERK) through a mechanism requiring calcium influx through L-type calcium channels in αT3-1 cells and primary rat gonadotropes. Further studies were undertaken to explore the signaling mechanisms by which the GnRH receptor is coupled to activation of another member of the MAPK family, c-Jun N-terminal kinase (JNK). GnRH induces activation of the JNK cascade in a dose-, time-, and receptor-dependent manner in clonal αT3-1 cells and primary rat pituitary gonadotrophs. Coexpression of dominant negative Cdc42 and kinase-defective p21-activated kinase 1 and MAPK kinase 7 with JNK and ERK indicated that specific activation of JNK by GnRH appears to involve these signaling molecules. Unlike ERK activation, GnRH-stimulated JNK activity does not require activation of protein kinase C and is not blocked after chelation of extracellular calcium with EGTA. GnRH-induced JNK activity was reduced after treatment with the intracellular calcium chelator BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester), whereas activation of ERK was not affected. Chelation of intracellular calcium also reduced GnRH-induced activation of JNK in rat pituitary cells in primary culture. GnRH-induced induction and activation of the JNK target c-Jun was inhibited after chelation of intracellular calcium, whereas induction of c-Fos, a known target of ERK, was unaffected. Therefore, although activation of ERK by GnRH requires a specific influx of calcium through L-type calcium channels, JNK activation is independent of extracellular calcium but sensitive to chelation of intracellular calcium. Our results provide novel evidence that GnRH activates two MAPK superfamily members via strikingly divergent signaling pathways with differential sensitivity to activation of protein kinase C and mobilization of discrete pools of calcium.

Mitogen-activated protein kinases (MAPKs) are regulated by various stimuli and are known to play critical roles in the control of multiple cell functions. Three major MAPK family members are known to exist: ERKs (p42 and p44 MAPKs), JNK, and p38 MAPK (1). ERKs are often activated by growth factors and have been shown to regulate growth and differentiation in many cells (2). JNK and p38 are often activated by stress stimuli such as ultraviolet irradiation or osmotic shock and, in many cases, inhibit cell growth or cause apoptosis (3). However, it is becoming increasingly more obvious that these three MAPKs have diverse functions in differentiated cells and that the balance of their concurrent activation may be regulated in a complex manner and instrumental in the control of differentiated cell function. Definition of MAPK signaling pathway architecture and regulation is necessary for examining the consequences of concurrent activation of multiple MAPK superfamily members.

G protein-coupled receptors activate MAPK signaling pathways through mechanisms that vary with specific ligand-receptor interactions, heterotrimeric G protein subtypes, and cellular phenotype (1). The hypothalamic decapeptide GnRH is coupled to concurrent activation of all three family members of the MAPK superfamily. Activation of the Gq/11-coupled receptor for GnRH in the clonal gonadotrope αT3-1 cell line or in pituitary cells in primary culture results in stimulation of a complex signaling cascade that includes activation of phospholipase C, production of IP3 and diacylglycerol, and subsequent activation of PKC (4, 5). In addition, GnRH receptor occupancy is linked with an increase in intracellular calcium through mobilization of two distinct pools in both αT3-1 cells and rat pituitary gonadotrophs (4–6). Extracellular calcium enters the cell through VGCCs in the plasma membrane while IP3 releases calcium from intracellular stores. The IP3-released calcium has been shown to be the critical signal required for secretion of the gonadotropic hormones, luteinizing hormone, and follicle-stimulating hormone (7, 8). Influx of calcium through L- and T-type VGCCs is initiated independently from the IP3-mediated signal, but calcium influx through the plasma membrane is ultimately required for replenishment of intracellular stores (9).

We have demonstrated recently that calcium influx through VGCCs is absolutely required for activation of ERK by the GnRH receptor agonist buserelin in both αT3-1 cells and pituitary cells maintained in primary culture (10). Further, stimulation of VGCCs was a sufficient signal for activation of ERK kinase(s); ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; GnRH, gonadotropin-releasing hormone; IP3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; VGC(s), voltage-gated calcium channel(s); PAK1, p21-activated kinase; MEK, MAPK kinase; PMA, phorbol 12-myristate 13-acetate; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N′,N′-tetraacetic acid acetoxymethyl ester; BIM, bisindolylmaleimide 1 (GF 109203X); BIM 5, bisindolylmaleimide 5; Me2SO, dimethyl sulfoxide; GST, glutathione S-transferase.
in the absence of hormone. IP3-released calcium did not appear to be involved in the signaling cascade linking the GnRH receptor to ERK activation. Activation of ERK by GnRH in αT3-1 cells required PKC (11, 12), and our results led to the hypothesis that the GnRH-induced VGCC signal required for activation of ERK may be located downstream of PKC activation (10). The goal of the experiments described here was to investigate possible differences in mechanisms integrating activation of the ERK and JNK pathways induced by GnRH receptor occupancy. We report that the signaling cascade linking the GnRH receptor to JNK may involve Cdc42-, PAK1- and MKK7-like molecules. In contrast to the signaling pathway for activation of ERK, GnRH-induced activation of JNK occurs independently of both PKC activation and extracellular calcium. Activation of JNK, but not ERK, by GnRH was inhibited by chelation of intracellular calcium. Inhibition of GnRH-induced JNK activity by chelation of intracellular calcium was also observed in studies using rat pituitary cells in primary culture. Our results support the conclusion that there is divergence in the signaling pathways coupling the GnRH receptor to multiple MAPKs with selective requirements for classical downstream signaling molecules as well as PKC and pharmacologically distinct calcium signals. Defining specific elements that are critical for activating MAPKs is fundamental to the understanding of GnRH-mediated regulation of immediate early and late response genes that control gonadotropin function.

MATERIALS AND METHODS

Cells and Tissue Culture—αT3-1 cells, an immortalized mouse pituitary cell line of the gonadotrope lineage, were cultured in monolayer in Dulbecco’s Modified Eagle’s medium supplemented with 5% fetal bovine serum and 5% horse serum (Life Technologies, Inc.). Cells were grown to approximately 70% confluence before use in studies. For kinase assays and immunoblot studies, cells were starved for 2 h before receiving hormone. Some experiments were carried out using EGF to chelate extracellular calcium. The ionic concentration of calcium chloride in Dulbecco’s modified Eagle’s medium is 1.8 mM. The GnRH agonist buserelin (1-α-Arg(Bu)6,Pro9-ethylamide) (GnRH) was applied to the cells at 10 nM for various lengths of time. Drugs (nifedipine, PMA, RAFTAM, H-89, staurosporine, BIM 1, and BIM 5) were prepared as stock solutions in DMSO, acetic acid, or ethanol and applied to the cells in Dulbecco’s modified Eagle’s medium. Cells were never exposed to >0.1% DMSO, acetic acid, or ethanol, and these concentrations of vehicle had no effect on responses of αT3-1 cells.

Pituitary cells for primary cultures were taken from the superior pituitary of the Harlan Sprague-Dawley rats. Pituitary cells were placed in filter-sterilized dissociation medium consisting of 137 mM NaCl, 25 mM Hepes, 1 mM KCl, 2 mM glucose, pH 7.3. The pituitaries were sliced into small fragments and digested with collagenase type II (1 mg/ml) and hyaluronidase type V (1 mg/ml) for 30 min at 37 °C. After digestion, cells were triturated, collected, and placed in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum and 5% horse serum. This procedure was repeated three times, and cells were collected after each trituration. Cells were collected on poly-l-lysine-coated dishes and maintained in culture for 48 h before treatment with hormone.

Antibodies, Immunoprecipitation, Immunoblotting, and Kinase Assays—For immunoprecipitations, cells were treated with drugs for specified time periods and then washed with ice-cold buffer containing 0.15 M NaCl and 10 mM Hepes (pH 7.5). The cells were lysed in a lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 2 mM EDTA, 5 mM sodium vandate, 5 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride on ice for 30 min at 37 °C. After centrifugation, the lysates were washed once in 1 ml of lysis buffer, twice in 1 ml of ice-cold wash buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA, 5 mM sodium vandate, 5 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride and once in 0.5 ml of a kinase buffer containing 20 mM Hepes (pH 7.5), 20 mM MgCl2, 25 mM β-glycerophosphate, 100 mM glycerol, 0.1 mM ATP, and 2 mM diethiothreitol. The reaction mixture (50 μl) contained the agarse beads suspended in kinase buffer, [γ-32P]ATP and substrate GST-ATF2 for JNK assay. Samples were subjected to kinase assay for 30 min at 30 °C with frequent mixing. After kinase assay the reaction was stopped with the addition of SDS loading buffer, then samples were boiled for 2 min, resolved by SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography. All of the experiments presented were conducted at least three times with equivalent results.

Plasmons and Transfection Experiments—The expression vector for Gal4 DNA binding domain c-Jun and the luciferase reporter containing 5 Gal4 DNA binding sites upstream of the E1B TATAA box and luciferase coding sequences have been described previously (13). MKK7 was a gift from Melanie Cobb (University of Texas, Southwestern), and Cdc42N17 was a gift from Dr. Rick Cerione (Cornell University). All plasmids used in transfection studies were prepared by centrifugation through cesium chloride using standard methods. Prior to all studies, cells were split to fresh medium and cultured to approximately 60–70% confluence. Transient transfections were accomplished using the calcium phosphate method (Life Technologies, Inc.) for Gal4-c-Jun studies, as there was a requirement for adherent cells immediately after transfection. Overexpression of dominant negative molecules was accomplished by electroporation as described previously (13). Briefly, for transient transfection studies with dominant negative and kinase-defective molecules, cells were transfected by electroporation using a single electrical pulse at 220 V and 950 microfarads. Some transfected cells received the calcium chelator BAPTA-AM (20 μM) approximately 8 h after electroporation. Cells were collected by scraping 6 h after the final administration of inhibitors, lysed by three freeze-thaw cycles, and luciferase activity was determined as described (13).

Electrophysiology—All whole cell recordings were made at room temperature. The whole cell perforated patch technique (14) was used. All recordings were performed 1–2 days after cells were plated. Patch clamp recordings were done on cells that were not in contact with other cells and had no cell processes to avoid possible cell-cell coupling artifacts and to maintain good space clamp. Patch clamp electrodes were made with soft capillary glass and adjusted to obtain a tip resistance of approximately 2–4 megohm. Amphotericin B (Sigma) was added from a fresh 1 mg/ml solution to obtain a final concentration of 20 μg/ml in a 1-mM square pulse of 10 ms at 10 Hz monitored the electrode tip resistance. Once a high resistance seal (>5 gigaohms) was formed between the recording pipette and the cell, the access resistance was monitored until it reached values less than 30 megohms. Capacitance was monitored before and after each experiment. All voltage clamp protocols were generated and currents recorded using an Axopatch 1-C amplifier and pClamp 5.51 acquisition system (Axon Instruments). The extracellular solution was exchanged continuously during recordings to add/wash out drugs. Bath perfusion was performed by exchanging the content of the 35-mm culture dish with recording solutions at a rate of ~2 ml/min using a gravity flow system. Drugs were added in the extracellular solution. For recording currents through VGCCs, barium was used as the charge carrier and the solutions were, in mM: external: 20 BaCl2, 109 N-methyl-D-glucamine, 25 KCl, 1 MgCl2, 10 Hepes, and 8 mM glucose (pH 7.2 with NaOH) and internal: 120 CsCl, 3 MgCl2, and 25 Hepes (pH 7.2 with CsOH). Currents were recorded from cells stepped for 90 ms from a holding potential of ~60 mV to test potentials between ~50 and +40 mV (10-mV increments).

References—(1) Cao, J., A. Surprenant, and D. K. Murphy. The SK3 splice variant is associated with the slow component of the T-type calcium current and is resistant to channel blocker toxins. J. Gen. Physiol. 111: 65–71, 1998.
**RESULTS**

**GnRH-induced Activation of JNK Is Dose-, Time-, and Receptor-dependent**—αT3-1 cells were treated with multiple doses of buserelin, a specific GnRH receptor agonist, to determine the optimal dose of buserelin for activation of JNK (Fig. 1A). Cell lysates were collected and assayed for JNK activity via an immune complex kinase assay using GST-ATF2 as a substrate. For all subsequent experiments, buserelin was administered at 10 nM. The time course of JNK activation was examined by stimulating αT3-1 cells with buserelin for multiple time points ranging from 15 min to 2 h. Buserelin stimulated an increase in JNK activity by 15 min, which persisted for up to 1 h and was decreased by 2 h (Fig. 1B). To provide evidence that GnRH receptor occupancy was required for JNK activation, the cells were pretreated with the specific GnRH receptor antagonist, antide. Antide was applied to αT3-1 cells 30 min before and during exposure to buserelin for 5, 15, or 30 min. Buserelin-induced JNK activity was blocked by antide (Fig. 1C), providing direct evidence that the observed buserelin-induced increase in JNK activity was GnRH receptor-mediated. Consistent with results observed in studies using αT3-1 cells, stimulation of rat pituitary cells in primary culture with buserelin for 0, 15, or 30 min induced a time-dependent increase in JNK activity (Fig. 1D). This observation suggests that the αT3-1 cell model accurately reflects fully differentiated pituitary cells in primary culture.

Cdc42-, PAK1-, and M KK7-like Molecules Are Involved in the Signaling Pathway Linking the GnRH Receptor to JNK—One reported signaling cascade leading to activation of JNK consists of Cdc42, PAK1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase, and JNK kinase (M KK7) (3, 15–17). To determine whether the buserelin signal to JNK involves these signaling molecules, αT3-1 cells were cotransfected with 1 μg of wild type JNK, 1 μg of wild type ERK2, and 5 μg of either dominant negative Cdc42 (Cdc42N17), truncated PAK1(1–231) molecule, or kinase-defective M KK7. Transfected cells were stimulated with buserelin for 0, 15, or 30 min, lysed, and divided for examination by kinase assay for JNK activity and by Western blot for ERK activation. Overexpression of either Cdc42 or PAK1 reduced buserelin-stimulated JNK activity but had no effect on activation of ERK (Fig. 2A). Overexpression of dominant negative M KK7 also reduced buserelin-induced JNK activity, whereas ERK activation was not influenced (Fig. 2B). Similar results were observed at higher doses of dominant negative or kinase-defective expression vectors (data not shown), suggesting that the doses used provided maximal responses.

**GnRH-stimulated Activation of ERK and JNK Exhibits Differential Sensitivity to PKC Inhibition**—αT3-1 cells express multiple PKC isozymes, including PKC-α, -ε, and -η (11, 18). Previous studies have suggested that activation of both GnRH-induced ERK and JNK in αT3-1 cells requires PKC (12, 13, 19). Initial studies comparing the PKC requirement for buserelin-induced ERK and JNK activation were performed using the PKC inhibitor BIM 1 (GF109203X) and its structurally related but functionally inactive negative control, BIM 5. Cells were pretreated for 30 min with control vehicle (Me₃SO), 2 μM BIM 1, or 2 μM BIM 5 before buserelin application for 0, 15, or 30 min. After hormone treatment cells were lysed and analyzed for JNK activity by kinase assay or ERK activation by Western blot. As expected, buserelin-induced ERK activation was reduced in the presence of BIM 1 and essentially unaffected by treatment with BIM 5 (Fig. 3A). Surprisingly, both BIM 1 and the negative control BIM 5 reduced buserelin-induced JNK activity (Fig. 3A). These results suggest that the reduction of JNK activity by the BIM drugs is likely not caused by PKC inhibition but by a nonspecific effect of the drugs. Therefore, contribution of PKC isozymes to JNK activation by GnRH was examined further by treating αT3-1 cells with 100 nM PMA for 16–20 h to deplete diacylglycerol-dependent PKC isozymes. Chronic treatment with PMA results in nearly a complete loss of detectable PKC-α and PKC-ε isozymes (18). Depletion of PKC isozymes in the current studies resulted in no obvious reduction in the magnitude of JNK activation at 15 and 30 min, whereas buserelin-induced activation of ERKs was blocked completely by PKC depletion at all time points (Fig. 3B). Similar results were obtained after treatment with the PKC inhibitor staurosporine (Fig. 3C) and H-89 at doses previously shown to inhibit PKC activity (20; data not shown). These results indicate that upstream effectors of ERK and JNK diverge at the level of PKC. PKC is absolutely required
for ERK activation, whereas activation of JNK is not affected remarkably by pharmacological inhibition of PKC inhibition or depletion of isozymes after chronic administration of PMA.

It has been shown that PMA increases the current flow through αT3-1 cell VGCCs at a magnitude similar to that of the current increase observed after GnRH treatment (21). One likely scenario is that buserelin-stimulated PKC may mediate an increase in current flow through VGCCs (10) in αT3-1 cells, as has been described in other cells (22, 23). We demonstrated in a recent report that treatment with the VGCC channel blocker nifedipine inhibits buserelin-induced activation of ERK but not JNK (10), and in the current paper we present evidence indicating that buserelin-induced ERK but not JNK activation requires PKC activity. To gain additional insight into the role of PKC in buserelin-stimulated cells, we performed a direct examination of the buserelin-induced increase in current influx through VGCCs in the absence or presence of the PKC inhibitor staurosporine using the perforated patch method of whole cell recording (Fig. 3D). Barium was used as the charge carrier, and the peak current was observed at −10 mV. The peak voltage-activated barium current in control cells was −62.9 ± 7.3 pA, whereas in the presence of 100 nM buserelin the peak current was −86.1 ± 6.8 pA (n = 10 cells). Our data are similar to results reported in a previous study examining GnRH-stimulated barium current through VGCCs using the standard method of whole cell recording (21). We report that buserelin induced an approximately 20–25% increase in the barium current through VGCCs. This increase was abolished completely in buserelin-stimulated αT3-1 cells pretreated for 30 min with 500 nM staurosporine (n = 4, Fig. 3D). Data shown are from a representative cell. In addition, we have shown that ERK can be activated by the VGCC activator Bay K 8644 in control or PKC-depleted cells, suggesting that PKC action is upstream of calcium influx through VGCCs (10). This evidence supports the hypothesis that PKC functions to mediate the buserelin-induced increase in calcium influx through VGCCs and provides additional insight into the mechanism by which the GnRH

**FIG. 2.** Overexpression of dominant negative Cdc42 or kinase-defective PAK or MKK7 reduces buserelin-stimulated JNK activity. Panel A, αT3-1 cells were cotransfected by electroporation with 1 μg of FLAG-JNK, 1 μg of ERK2, and either 10 μg of control plasmid (pcDNA3), dominant negative Cdc42, or kinase-defective PAK. 18 h later buserelin was administered for 0, 15, or 30 min, and cell lysates were collected and analyzed for JNK activity by immunoprecipitation followed by kinase assay or for ERK activity by Western blotting using an antibody for phospho-ERK. For all of the experiments the blots were stripped and reprobed with ERK antibody demonstrating equal protein amounts in each lane. Panel B, αT3-1 cells were cotransfected by electroporation with 1 μg of FLAG-JNK, 1 μg of ERK2, and either 10 μg of control plasmid (pcDNA3) or kinase-defective MKK7. 18 h later buserelin was administered for 0, 15, or 30 min, and cell lysates were collected and analyzed for JNK activity by immunoprecipitation followed by kinase assay or for ERK activity by Western blotting using an antibody for phospho-ERK. For all of the experiments the blots were stripped and reprobed with ERK antibody demonstrating equal protein amounts in each lane.

**FIG. 3.** Effects of PKC down-regulation on buserelin-stimulated JNK activation. Panel A, αT3-1 cells were treated with the PKC inhibitor BIM 1 (GP 109203X, 2 μM) or the structurally related but functionally inactive BIM 5 (2 μM) for 30 min before stimulation with buserelin for 0, 15, or 30 min. After treatment, cell lysates were collected and analyzed for JNK activity by immunoprecipitation followed by kinase assay or for ERK activity by Western blotting using an antibody for phospho-ERK. Panel B, αT3-1 cells were exposed to chronic (approximately 16 h) treatment with 100 nM PMA or control vehicle (Me2SO) before stimulation with 10 nM buserelin for 0, 15, or 30 min. After treatment, cell lysates were collected and analyzed for JNK activity by immunoprecipitation followed by kinase assay or for ERK activity by Western blotting using an antibody for phospho-ERK. For all of the experiments the blots were stripped and reprobed with ERK antibody demonstrating equal protein amounts in each lane.
receptor is coupled to ERK, but not JNK, activation.

**GnRH-induced Activation of JNK Is Not Inhibited by Acute Chelation of Extracellular Calcium**—Activation of the GnRH receptor results in IP3-mediated increases in intracellular calcium which have been shown to be the primary signal for exocytosis in αT3-1 cells and primary gonadotropes (7–9). Calcium influx through the plasma membrane is thought to play a role in maintaining intracellular calcium stores. Previous studies (9) have indicated that αT3-1 cells maintained in calcium-free medium for longer than 20 min undergo a time-dependent depletion or rundown of internal IP3-sensitive calcium stores. Therefore, accurate examination of a requirement for extracellular calcium was accomplished by rapidly chelating extracellular calcium and subsequently measuring JNK activity after brief exposures to buserelin. Treatment of cells with 5 mM (Fig. 4) or 15 mM EGTA (data not shown) for 2 min before stimulation with buserelin for 2, 5, or 10 min did not block GnRH-induced JNK activity but inhibited buserelin-induced ERK activation completely. These data demonstrate unequivocally that there is divergence in the signaling pathways coupling the GnRH receptor to activation of ERK and JNK (Fig. 4).

**BAPTA-AM Reduces GnRH-stimulated JNK Activity**—To investigate further a requirement for intracellular calcium mobilization in buserelin-stimulated JNK activity, αT3-1 cells were pretreated with control vehicle (MeSO) or the calcium chelator BAPTA-AM for 30 min before exposure to buserelin for 0, 15, or 30 min. After hormone stimulation, cells were lysed, and cell lysates were examined for JNK activity (kinase assay) or ERK activation (Western blot). Pretreatment with either 10 or 50 μM BAPTA-AM greatly reduced buserelin-induced JNK activity but had only a slight effect on ERK activation (Fig. 5A).

Although BAPTA-AM is a known and commonly used chelator of intracellular calcium, it can have varying degrees of effectiveness depending on cell type and experimental conditions. An examination of indo-1 fluorescence observed upon buserelin stimulation after BAPTA-AM treatment suggested that the spike phase of the fluorescence signal, corresponding to IP3-released calcium, was reduced (Fig. 5B). In contrast, the plateau phase, corresponding to calcium entry through VGCCs, was still quite prominent in the BAPTA-loaded cells (Fig. 5B). The buserelin-stimulated indo-1 fluorescence in cells pretreated with BAPTA-AM is greatly reduced in cells that have also been treated with nifedipine (Fig. 5C). These results suggest that in the buserelin-stimulated αT3-1 cell, BAPTA-AM

---

2 J. M. Mulvaney and M. S. Roberson, unpublished observations.
effectively chelates calcium released from internal stores by IP3 (spike phase) but is not as effective at chelating calcium entering the cell through VGCCs (plateau phase).

We report above that buserelin induces JNK activity in rat pituitary cells maintained in primary culture. To determine whether a requirement for intracellular calcium mobilization exists for buserelin-induced JNK activation in cultured rat pituitary cells, cells were pretreated with control vehicle (Me₂SO) or the calcium chelator BAPTA-AM for 30 min before exposure to buserelin for 0, 15, or 30 min. After hormone stimulation, cells were lysed and cell lysates examined for JNK activity (kinase assay). A portion of the immunoprecipitate was reserved for examination of the amount of JNK protein (Western blot). Pretreatment with BAPTA-AM inhibited buserelin-induced JNK activity completely (Fig. 5D).

**BAPTA-AM Pretreatment Blocks Buserelin-induced Activation of JNK Targets, but ERK Targets Are Unaffected**—It has been shown that stimulation of αT₃-1 cells with GnRH results in increased mRNA and protein for the immediate early genes c-fos and c-jun (10, 24). To examine for effects of BAPTA-AM on downstream targets of GnRH-induced MAPKs, αT₃-1 lysates were examined for the amounts of c-Fos and c-Jun protein after 1 or 2 h of hormone stimulation after a 30-min pretreatment with control vehicle or BAPTA-AM. Buserelin-induced increases in c-Fos protein amounts were unaffected in the BAPTA-AM-treated cells, whereas c-Jun protein amounts and phosphorylation, as measured by retarded electrophoretic mobility shift, were reduced in BAPTA-AM-treated cells compared with control (Fig. 6A). An alternative strategy for examining the sensitivity of c-Jun activation to intracellular calcium was to cotransfect αT₃-1 cells with a Gal4-c-Jun fusion protein and a Gal4-dependent luciferase reporter and examine buserelin-stimulated luciferase activity in the absence and presence of BAPTA-AM. These studies revealed that buserelin-stimulated c-Jun transcriptional activity was dramatically inhibited by pretreatment with BAPTA-AM (Fig. 6B).

**DISCUSSION**

It is becoming increasingly evident that the specific nature of the way extracellular signals are interpreted within a cell to regulate gene transcription can vary greatly. Outcomes can vary depending upon cell type, the type of stimulus and the duration and intensity of the stimulus, as well as influences from other signaling pathways that may be concurrently activated. The mechanisms by which an extracellular signal uses ubiquitous intracellular signaling molecules to elicit highly specific cellular responses are not well understood. The goal of the present studies was to enhance our knowledge and understanding of the mechanisms linking the GnRH receptor to multiple signaling pathways that influence gene transcription. Such information is critical for understanding how GnRH regulates gonadotrope function and exerts control over key reproductive processes.

GnRH receptor occupancy results in simultaneous activation of multiple MAPK superfamily members (11–13, 18, 19, 25). These observations are consistent with ligand activation of the endothelin B, α₁a-adrenergic, and angiotensin II G protein-coupled receptors (26–29). Although it is known that buserelin activates JNK in αT₃-1 cells (25), we believe that data presented here provide the first demonstration of JNK activation coupled to GnRH receptor stimulation in rat pituitary cells in primary culture. Translational studies are essential in that they serve to reinforce the fidelity and appropriateness of the αT₃-1 cell model for the study of GnRH receptor-linked signal transduction.

Results from the present studies support the conclusion that signaling pathways linking the GnRH receptor to activation of ERK and JNK in αT₃-1 cells diverge at the level of PKC. Although diacylglycerol-dependent PKC isozymes are absolutely required for ERK and p38 MAPK activation (12, 13, 18), GnRH-induced JNK activation in αT₃-1 cells occurs via a PKC-independent pathway. This finding is similar to results reported for the Goq/11-coupled angiotensin II receptor, which is coupled to JNK in a PKC-independent manner in rat liver epithelial cells as well as hypothalamic and brainstem neurons (29, 30). However, angiotensin II receptors are coupled to JNK in a PKC-dependent mechanism in cardiac myocytes (31), demonstrating the variability of signaling pathways in heterologous cells. We cannot exclude the possibility that a phorbol ester-insensitive form of PKC, such as PKC-ε (32), may play a role in GnRH-induced JNK activation. Examination of this possibility awaits development of potent inhibitors that have specificity for individual PKC isozymes.

We have reported recently that activation of ERK (but not JNK) by GnRH in clonal and primary gonadotropes requires calcium entry though VGCCs (10). It has been demonstrated previously in αT₃-1 cells that PMA stimulates an increase in current flow though VGCCs which is similar to the current observed after treatment with GnRH (21). In this study we provide direct electrophysiological evidence that the GnRH-induced VGCC signal is blocked when cells have been treated with the PKC inhibitor staurosporine. If PKC is functioning to facilitate the buserelin-induced increase in current flux...
through VGCCs, and JNK does not require PKC activation, it is not surprising that buserelin-stimulated JNK activity was not reduced by treatment with the VGCC antagonist nifedipine. These data lend support to our conclusion that buserelin-stimulated JNK activity in αT3-1 cells requires neither PKC nor calcium entry through VGCCs.

Pharmacological blockade of t-type VGCCs had no influence on buserelin-induced JNK activity (10), and JNK catalytic activity was still observed after chelation of extracellular calcium with EGTA. BAPTA-AM-treated cells exhibited reduced JNK activity but normal ERK activity. An examination of the fluorescence profile of BAPTA-AM-treated cells indicated that the rapid spike phase of the response, corresponding to release of calcium from IP3-gated intracellular stores, was reduced, whereas the VGCC plateau phase was still quite robust. These results, along with those obtained from previous studies examining the calcium requirement for ERK activation, indicate that the GnRH receptor makes use of two different and discrete calcium signals for activation of ERK and JNK. We suggest that because of the discrete nature of these calcium signals, it is likely that they are confined to distinct subcellular compartments.

It is presently not clear how the IP3-released calcium signal influences the signaling pathway linking the GnRH receptor to JNK. Dominant negative PAK1, which we have suggested may be important in linking the GnRH receptor to JNK, markedly reduced JNK activation by angiotensin II in Chinese hamster ovary and COS cells expressing the angiotensin II type 1 receptor (33). Further, angiotensin II-mediated activation of PAK1 and JNK was inhibited by chelation of intracellular calcium with BAPTA-AM (33), suggesting that PAK1 itself or another upstream signaling molecule was calcium-sensitive. Angiotensin II has been shown to stimulate activation of the proline-rich tyrosine kinase in cultured vascular smooth muscle cells via a mechanism that requires release of calcium from internal stores (34). The authors also reported that angiotensin II was associated with proline-rich tyrosine kinase-Src complex formation (34). A recent report by Levi et al. (19) suggested that Src is required for GnRH-induced JNK activity in αT3-1 cells. Future experiments will aim at addressing the potential involvement of additional JNK-related signaling molecules as well as investigating the specific sites for calcium regulation for GnRH-stimulated MAPK activity.

Data presented here and in a previous study (10) indicate that the requirement for specific GnRH-stimulated calcium signals affects the downstream targets c-Fos and c-Jun via a MAPK-specific mechanism. Concurrent activation of multiple MAPKs by GnRH could have varying effects on generation of transcriptionally active AP-1 heterodimers formed by the dimerization of c-Fos and c-Jun, possibly enhancing the ability of AP-1 to bind to its target genes and regulate numerous cellular processes. The role and importance of MAPK signaling and AP-1 in the regulation of gonadotrope cell function and GnRH-dependent gene expression have recently been the focus of a detailed study of the GnRH receptor gene (35). Expression of the GnRH receptor gene requires a putatively tissue-specific tripartite enhancer consisting of a steroidogenic factor 1 binding site, an element required for tissue-specific expression, and a consensus AP-1 site. Disruption of the AP-1 site by mutagenesis resulted in a blockade of GnRH inducibility of a GnRH receptor promoter reporter gene (35). Little is known about the ability of GnRH-induced JNK to influence transcription of the GnRH receptor gene. It is possible that through the use of discrete calcium signals and multiple MAPKs the GnRH receptor possesses an exquisitely sensitive mechanism by which the immediate early genes c-fos and c-jun can be regulated as well as multiple late response genes that have an AP-1 site for regulation of transcription, such as the GnRH receptor gene.

Data from the present studies, combined with our recent findings regarding buserelin-induced activation of ERK (10), support the following mechanistic model for activation of ERK and JNK by the GnRH receptor (Fig. 7). The relevance of the model is supported by critical parallel studies done with rat pituitary cells in primary culture. After ligand binding of the GnRH receptor, activation of ERK is dependent upon diacylglycerol-sensitive PKC isoforms and requires calcium influx though plasma membrane VGCCs. We have demonstrated that activation of the ERK signaling pathway involves activation of Raf kinase and does not appear to require release of calcium from intracellular stores. We also suggest that PKC functions to facilitate GnRH-induced calcium flux through VGCCs. In contrast, activation of JNK by the GnRH receptor does not require PKC or calcium entry through VGCCs but appears to require release of calcium from internal stores. JNK activation occurs via a pathway that involves Cdc42-, PAK-, and MKK7-like signaling molecules. These data are in agreement with a previously reported role for Cdc42 (19) in the GnRH to JNK cascade and provide new evidence for the involvement of two additional signaling molecules, PAK1 and MKK7, in the GnRH pathway.

The sensitivity of JNK activation to intracellular calcium fluctuations suggests that release of calcium from IP3-gated intracellular stores may have functions in addition to the widely accepted role as being the primary signal for hormone secretion. We suggest that in addition to serving as a trigger for hormone release, GnRH-induced mobilization of intracellular calcium may exert some control over JNK-related nuclear events. Further studies are required to determine how the two different MAPK pathways induced by ligand binding to the GnRH receptor have such selective requirements for distinct calcium signals. In addition, defining the roles of calcium-sensitive ERK and JNK activity upon transcription of immediate early and late response genes in gonadotropes will be critical for understanding how GnRH manages reproductive processes.

Acknowledgments—We are grateful to Tong Zhang for critical review of this manuscript and to Dr. Clare Fewtrell for assistance with the fluorometry experiments. We extend thanks to Dr. Lynn Heasley, Dr. Melanie Cobb, and Dr. Richard Cerione for generously supplying plasmids for MKK7, PAK, and Cdc42. We also thank Sharon Guest-Tagliavento for excellent technical assistance.
REFERENCES

1. Cobb, M. H. (1999) *Prog. Biophys. Mol. Biol.* 71, 479–500
2. Robinson, M. J., and Cobb, M. H. (1997) *Curr. Opin. Cell Biol.* 9, 180–186
3. Minden, A., and Karin, M. (1997) *Biochem. Biophys. Acta* 1333, F85–F104
4. Horn, F., Bilezikjian, L. M., Perrin, M. H., Bosma, M. M., Windle, J. J., Huber, K. S., Blount, A. L., Hille, B., Vale, W., and Mellon, P. L. (1991) *Mol. Endocrinol.* 3, 347–353
5. Stojilkovic, S. S., Reinhart, J., and Catt, K. J. (1994) *Endocr. Rev.* 15, 462–499
6. McArden, C. A., Bunting, R., and Mason, W. T. (1992) *Mol. Cell. Neurosci.* 3, 124–132
7. Martian, J., and Conn, P. M. (1995) *Mol. Pharmacol.* 46, 205–217
8. Naor, Z., Capponi, A. M., Rossier, M. F., Ayalon, D., and Limor, R. (1988) *Mol. Endocrinol.* 2, 512–520
9. Hille, B., Tse, A., Tse, F. W., and Bosma, M. M. (1995) *Recent Prog. Horm. Res.* 50, 75–95
10. Mulvaney, J. M., Zhang, T., Fewtrell, C., and Roberson, M. S. (1999) *J. Biol. Chem.* 274, 29796–29804
11. Sundaresan, S., Colín, I. M., Pestell, R. G., and Jameson, J. L. (1996) *Endocrinology* 137, 304–311
12. Reiss, N., Llevi, L. N., Shacham, S., Harris, D., Seger, R., and Naor, Z. (1997) *Endocrinology* 138, 1673–1682
13. Roberson, M. S., Miau-Press, A., Laurance, M. E., Stork, P. J., and Maurer, R. A. (1995) *Mol. Cell. Biol.* 15, 3531–3539
14. Horn, R., and Marty, A. (1988) *J. Gen. Physiol.* 92, 145–159
15. Cose, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) *Cell* 81, 1137–1146
16. Manser, E., Leung, T., Sahlin, H., Zhao, Z., and Lim, L. (1994) *Nature* 367, 40–46
17. Gaviria, T. P., Johnson, G. M. L. (1999) *Curr. Opin. Cell Biol.* 11, 211–218
18. Roberson, M. S., Zhang, T., Li, H. L., and Mulvaney, J. M. (1999) *Endocrinology* 140, 1310–1318
19. Lenvi, N. L., Hanoch, T., Benard, O., Rozenblatt, M., Harris, D., Reiss, N., and Naor, Z. (1998) *Endocrinology* 139, 215–224
20. Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Urito, K., Toshioka, T., and Hidaka, H. (1990) *J. Biol. Chem.* 265, 5267–5272
21. Bosma, M. M., and Hille, B. (1999) *Endocrinology* 130, 3411–3420
22. Izumi, S., Stojilkovic, S. S., Iida, T., Krmanovic, L. Z., Omelet, R. J., and Catt, K. J. (1990) *Biochem. Biophys. Res. Commun.* 170, 359–367
23. Groschner, K., Schuhmann, K., Meikes, G., Baumgartner, W., and Romanin, C. (1996) *Biochem. J.* 318, 513–517
24. Cesnjaj, M., Catt, K. J., and Stojilkovic, S. S. (1991) *Endocrinology* 134, 692–701
25. Mitchell, R., Foo, P. J., Leslie, T., Johnson, M. S., and Thomson, F. J. (1994) *J. Endocrinol.* 140, R15–R18
26. Aquilla, E., Whelchel, A., Knoll, H. J., Nelson, M., and Posada, J. (1996) *J. Biol. Chem.* 271, 31572–31579
27. Williams, N. G., Zhou, H., and Minneman, K. P. (1998) *J. Biol. Chem.* 273, 24624–24632
28. Duff, J. L., Berk, B. C., and Corson, M. A. (1992) *Biochem. Biophys. Res. Commun.* 188, 257–264
29. Zohn, I. E., Yu, H., Li, X., Cox, A. D., and Earp, H. S. (1995) *Mol. Cell. Biol.* 15, 6160–6168
30. Huang, X. C., Deng, T., and Sumners, C. (1998) *Endocrinology* 139, 245–251
31. Kudoh, S., Komura, I., Mizuno, T., Yamakazi, T., Zou, Y., Sato, M., Takekoshi, N., and Yakagi, Y. (1997) *Circ. Res.* 80, 139–146
32. Kratzmeier, M., Poch, A., Mukhopadhyay, A. K., and McArdle, C. A. (1996) *Mol. Cell. Endocrinol.* 118, 213–219
33. Schmitz, U., Ishida, T., Ishida, M., Suraphitach, J., Hasham, M. I., Pech, S., and Berk, B. C. (1998) *Circ. Res.* 82, 2672–2677
34. Sabri, A., Govindarajan, G., Griffin, T. M., Broyon, K. L., Sammar, A. M., and Lucchesi, P. A. (1998) *Circ. Res.* 83, 841–851
35. White, B. R., Durval, D. L., Mulvaney, J. M., Roberson, M. S., and Clay, C. M. (1999) *Mol. Endocrinol.* 13, 568–577
Divergent Signaling Pathways Requiring Discrete Calcium Signals Mediate Concurrent Activation of Two Mitogen-activated Protein Kinases by Gonadotropin-releasing Hormone
Jennifer M. Mulvaney and Mark S. Roberson

J. Biol. Chem. 2000, 275:14182-14189.
doi: 10.1074/jbc.275.19.14182

Access the most updated version of this article at http://www.jbc.org/content/275/19/14182

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 12 of which can be accessed free at http://www.jbc.org/content/275/19/14182.full.html#ref-list-1