Calcium Influx through L-type Channels Is Required for Selective Activation of Extracellular Signal-regulated Kinase by Gonadotropin-releasing Hormone*

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The hypothalamic decapeptide gonadotropin-releasing hormone stimulates mobilization of two discrete pools of calcium in clonal (αT3-1) and primary pituitary gonadotropes. A multidisciplinary approach was implemented to investigate the effects of discrete calcium fluctuations on the signaling pathways linking the gonadotropin-releasing hormone receptor to activation of mitogen-activated protein kinases and immediate early genes. Blockade of calcium influx through nifedipine-sensitive voltage-gated calcium channels reduced buserelin-induced activation of extracellular signal-regulated kinase (ERK) and c-Fos while activation of c-Jun N-terminal kinase and c-Jun was unaffected. Inhibition of buserelin-stimulated ERK activity by nifedipine was also observed in rat pituitary cells in primary culture. Direct activation of αT3-1 cell L-type calcium channels with the agonist Bay-K 8644 resulted in phosphorylation of ERK and induction of c-Fos. However, simple voltage-induced channel activation did not produce a sufficient calcium signal, since depolarization with 35 mM KCl failed to induce activation of ERK. Depletion of intracellular calcium stores with thapsigargin did not affect buserelin-induced ERK activation. An inhibitor of protein kinase C decreased calcium influx through nifedipine-sensitive calcium channels and phosphorylation of ERK induced by buserelin. Pharmacological inhibition of protein kinase C did not block Bay-K 8644-induced ERK activation. These observations suggest that calcium influx through L-type channels is required for GnRH-induced activation of ERK and c-Fos and that the influence of calcium lies downstream of protein kinase C.

Gonadotropin-releasing hormone (GnRH) is a hypothalamic decapeptide critical for normal mammalian reproductive development and function. Upon binding to its heterotrimeric G protein-coupled receptor in the plasma membrane of anterior pituitary gonadotropes, GnRH initiates a complex and diverse cascade of signaling events that regulates multiple cellular functions. Activation of the Goαq-coupled GnRH receptor results in phospholipase C-mediated generation of IP3 and diacylglycerol. GnRH stimulation of IP3 accumulation has been shown to be essential for the generation of intracellular calcium oscillations and concurrent secretion of the gonadotropic hormones luteinizing hormone and follicle-stimulating hormone (1). GnRH also mediates an influx of calcium through VGCCs in the gonadotrope plasma membrane. This influx of calcium appears to be independent of the IP3-mediated calcium event in that the two events can be blocked independently (2, 3). However, a long term interdependence of these two calcium pools has been described in that calcium influx is ultimately necessary for maintaining IP3-releasable stores (1). The ability of GnRH to mobilize discrete calcium pools has interesting implications for modulation of signal transduction pathways. Calcium is a ubiquitous signaling molecule that plays a crucial role in regulating signal transduction in many cell types (4). In some systems, cytoplasmic rises in intracellular calcium are pivotal for regulation of gene expression (5, 6). These studies have indicated that the spatial localization of intracellular calcium fluctuations is critical for determining how particular regulatory elements within genes will be influenced (7). A recent review discussed the fundamental importance of elementary or local calcium signals versus global calcium oscillations or waves as signals for control of specific cell functions in nonendocrine cells such as myocytes and neurons (8).

We have found that αT3-1 cells, a gonadotrope-derived cell line endogenously expressing GnRH-R, provide a unique and invaluable model system for investigating how signaling pathways may be regulated by discrete localized fluctuations in cell calcium. Stimulation of αT3-1 cells with the GnRH-R agonist buserelin results in activation of three members of the MAPK family (9–14), increased mRNA levels of the IEGs c-fos and c-jun (15), transcriptional activation of the gene for the common α-subunit of luteinizing hormone and follicle-stimulating hormone (16), and activation of the transcription factor Elk 1 (9). Previous studies have shown that activation of a MAPK family member, ERK, is absolutely required for buserelin-induced transcription of the α-subunit and the GnRH receptor gene (9, 17). Following exposure of αT3-1 cells to GnRH, a biphasic calcium response consisting of an initial IP3-dependent spike phase followed by a sustained extracellular calcium-requiring plateau phase is observed (2). The two discrete GnRH-induced calcium signals are subject to differential isolation or modulation by various pharmacological tools.

An earlier study demonstrated that in the absence of extracellular calcium, GnRH-stimulated MAPK activity was significantly reduced, while globally increasing intracellular calcium with a calcium ionophore had little or no effect on stimulation.
of MAPKs in the absence of GnRH (11). These results led the authors to conclude that calcium is necessary but not sufficient for stimulation of MAPK activity in αT3-1 cells. In a separate study, Cesnjaj et al. (15) reported that increased intracellular calcium appears to be sufficient to induce increased mRNA for the MAPK substrates c-Fos and c-Jun, while removal of extracellular calcium significantly enhanced GnRH-induced increases in message for these IEGs in αT3-1 cells. This would suggest that there may be differential effects of localized calcium on mRNA for specific GnRH-regulated target genes. Further, a recent study suggested that specific fluctuations in cell calcium may be important in the regulation of α-subunit gene transcription (18). However, the mechanism by which calcium may influence GnRH-stimulated α-subunit production was not resolved. While these studies have provided intriguing information about the importance of calcium at various points in the GnRH signaling pathway, the nature of the specific roles of spatial and temporal calcium signals in regulation of GnRH-stimulated MAPK activity, IEG induction, and α-subunit gene transcription remains unclear. Clearly, a careful examination of the requirement for discrete calcium pools in the signaling pathways linking the GnRH receptor to increased MAPK activity and subsequent induction of c-Fos and c-Jun protein amounts is warranted to enhance our understanding of the role of calcium in GnRH-R-linked signaling pathways leading to regulation of gene expression.

We have developed a multidisciplinary approach that enables us to investigate the potential interactions between calcium influx through VGCCs or IP3-released calcium and GnRH-stimulated MAP kinase signaling pathways and immediate early gene induction. We find that a differential sensitivity to calcium exists for GnRH induction of the MAP kinases ERK and JNK and the immediate early genes c-fos and c-jun. The results of studies presented here indicate that a specific signal involving calcium influx through L-type VGCCs is required for GnRH-induced activation of ERK and subsequent c-Fos induction, while fluctuations in IP3-released calcium do not appear to be involved. In addition, we report that specific activation of L-type VGCCs by Bay-K 8644 is sufficient stimulus for activation of the ERK pathway. However, increasing cytoplasmic calcium with elevated potassium or thapsigargin does not induce ERK phosphorylation. By combining pharmacological and fluorescence techniques, we are able to characterize the type of calcium signal required for activation of ERK.

Further, our data suggest that the effect of calcium is located downstream of PKC and upstream of Raf kinase in the signaling pathway linking the GnRH receptor to ERK activation.

**EXPERIMENTAL PROCEDURES**

**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 prior to lysis. For kinase assays and immunoblot studies, cells were serum-starved for 2 h before receiving hormone. Some experiments were carried out using modified physiological saline solutions. The standard solution used contained 127 mM NaCl, 1.8 mM CaCl2, 5 mM KCl, 2 mM MgCl2, 10 mM HEPES, 10 mM glucose, pH 7.4. For some experiments, barium or magnesium ions were substituted for calcium. For potassium depolarization experiments, the potassium concentration was increased to 35 mM, while the sodium concentration was decreased proportionally to maintain the osmolarity of the solution. The ionic concentrations of calcium chloride (1.8 mM) and potassium chloride (5 mM) in Dulbecco’s modified Eagle’s medium are the same as those present in the standard physiological saline solution. The GnRH agonist buserelin ([α-SE-R(But)2,Pro2-ethylamid–(GnRH)] was applied to the cells at 10 nM for various lengths of time. Drugs (nifedipine, Bay-K 8644, PD98059, PMA, H-89, staurosporine, GF 109203X, thapsigargin, Rp-cAMPS) were prepared as stock solutions in Me2SO, acetone, or ethanol and applied to the cells 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 following each trituration. Cells were plated on poly-L-lysine-coated dishes and maintained in culture for 48 h prior to treatment with hormone.

**Antibodies, Immunoprecipitation, Immunoblotting, and Kinase Assays—** For immunoprecipitations and Western blotting, 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 radioimmunoprecipitation 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 vanadate, 5 mM benzamide, and 1 mM phenylmethylsulfonyl fluoride on ice for 10 min. The cell lysates were collected and cleared by centrifugation. For Western blotting, proteins were resolved using SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane by electroblotting. Polyclonal (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and phosphospecific (New England Biolabs) antibodies to ERKs were used according to the manufacturers’ instructions. Immunostained proteins were visualized using enhanced chemiluminescence reagents (NEL Life Science Products). c-Fos and c-Jun antisera were obtained from Santa Cruz Biotechnology. The c-Fos antibody is specific to p62 c-Fos and, according to the manufacturer, is not cross-reactive with other Fos family members. Polyvinylidene difluoride membranes were stripped by soaking for 30 min at 55 °C in a solution containing 62.5 mM Tris (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol. JNK was immunoprecipitated by adding JNK-1 antibody (0.5 μg; Santa Cruz Biotechnology) and 25 μl of protein G- and A-agarose beads to clarified cell lysates. Samples were gently rotated for 2 h at 4 °C. The beads were washed twice in 1 ml of radioimmune precipitation buffer; twice in 1 ml of ice-cold Nonidet P-40 wash buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA, 5 mM sodium vanadate, 5 mM benzamide, and 1 mM phenylmethylsulfonyl fluoride; and once in 0.5 ml of kinase buffer containing 20 mM HEPES (pH 7.5), 20 mM MgCl2, 25 mM β-glycerophosphate, 100 mM sodium vanadate, 20 μM ATP, and 2 mM dithiothreitol. The reaction mixture (50 μl) contained the agarose beads suspended in kinase buffer, [γ-32P]ATP, and substrate GST-AKT2 (for JNK assay) or GST-Elk 1 (for ERK assay). Samples were incubated for 30 min at 30 °C with frequent mixing. Cells used for immunoprecipitations were lysed in radioimmune precipitation buffer containing 25 mM β-glycerophosphate for approximately 3 h at 4 °C. Raf was immunoprecipitated from clarified lysates by adding 2 μg of Raf-1 antibody (Santa Cruz Biotechnology) and 30 μl of protein G- and A-agarose beads and rocking at 4 °C overnight. The beads were washed twice in 1 ml of radioimmune precipitation buffer, three times in 1 ml of ice-cold Nonidet P-40 wash buffer and once in 0.5 ml of Raf kinase buffer containing 30 mM HEPES (pH 7.4), 7 mM MgCl2, 5 mM MgCl2, 1 mM dithiothreitol, and 15 μM ATP. Samples were incubated for 45 min at 30 °C with frequent mixing. The reaction mixture (50 μl) contained the agarose beads suspended in Raf kinase buffer, [γ-32P]ATP, and 10 μg of myelin basic protein. Following JNK, ERK, or Raf kinase assays, the reactions were stopped with the addition of SDS loading buffer, and 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.

**Plasmids and Transfection Experiments—** The expression vector for Gal4 DNA binding domain-Erk 1 transactivation domain and the luciferase reporter containing five Gal4 DNA binding sites upstream of the E1B TATA box and luciferase sequences was described (9). 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 media and cultured to approximately 60–70% confluence. All transient transfection studies were conducted as described previously (9). Briefly, for transient transfection studies, cells were transfected by electroporation using a single
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electrical pulse at 220 V and 950 microfarads. Some transfected cells either received the specific MEK1 inhibitor, PD98059 (50 μM), or the L-type calcium channel blocker, nifedipine (1 μM), approximately 8 h following electroporation. Inhibitors were added again approximately 16 h following electroporation. Cells were collected by scraping 6 h following the final administration of inhibitors and lysed by three freeze thaw cycles, and luciferase activity was determined as described (9).

**Fluorescence**—αT3-1 cells were trypanosized and resuspended in the standard physiological saline solution (see above) at a density of 10^6 cells/ml. Cells were loaded with 2 μM indo-1/AM (purchased from Molecular Probes, Inc., Eugene, OR) for 30 min at 37 °C in the presence of 0.1% bovine serum albumin. After loading, cells were washed and stored within 2 h. 3-ml aliquots of cell suspension were placed in acryl cuvettes and maintained at 37 °C with constant stirring. For some experiments, an initial volume of 2.2 ml of cells/cuvette was used, and a base-line fluorescence signal was established prior to the addition of 800 μl of control solution or an isotonic potassium chloride solution. For cuvettes receiving the potassium chloride solution, the final potassium concentration was 35 mm. Indo-1/AM fluorescence at 405 nm was monitored for measurement of free ionized calcium with a Perkin-Elmer LS-5 fluorescence spectrophotometer. Indo-1/AM was excited at 355 nm. All of the experiments presented were conducted at least three times with equivalent results.

**RESULTS**

**Nifedipine Differentially Blocks Buserelin-induced Activation of MAPKs and Induction of Immediate Early Genes**—Stimulation of αT3-1 cells with the GnRH agonist buserelin results in IP3-mediated mobilization of intracellular calcium as well as calcium entry through L-type and T-type VGCCs in the plasma membrane (1). Therefore, αT3-1 cells exhibit a biphasic calcium response following buserelin application. Imaging experiments have demonstrated that the calcium response consists of an extracellular calcium-independent IP3-mediated spike followed by a sustained plateau phase that is dependent upon extracellular calcium (2). An examination of the effects of buserelin on cytosolic calcium in the absence or presence of nifedipine confirms that the plateau phase is abolished in the nifedipine-treated cells while the spike phase remains (Fig. 1A). These initial studies verified the efficacy of nifedipine and enabled us to be confident in our subsequent pharmacological approach.

Experiments were performed to examine the role of calcium influx through VGCCs in buserelin-stimulated activation of two members of the MAPK family, ERK and JNK. αT3-1 cells pretreated with control vehicle (0.1% acetone) or the specific L-type calcium channel antagonist, nifedipine (1 μM), were exposed to buserelin for 15 or 30 min (Fig. 1B). Cell lysates were examined by Western blotting for the absence or presence of the dual phosphorylated (activated) form of ERK. The blots were then stripped and reprobed with antibody to total ERK to confirm equivalent sample loading. JNK activity was measured by kinase assay using the same cell lysates to be probed with c-Jun antibody. Cells treated with 10 nM buserelin, a GnRH receptor agonist, for 0, 15, or 30 min in the absence or presence of 1 μM nifedipine prior to collecting whole cell lysates for immunoprecipitation with JNK1 antibody and analysis by Western blotting using an antibody for phospho-JNK (p-JNK). ERK and JNK activity were analyzed by Western blotting using GST-ATF2 as a substrate. Western blots with an antibody to ERK were used to demonstrate equivalent protein amounts present in immunoprecipitates. αT3-1 cells were treated with 10 nM buserelin for 0, 60, or 120 min in the absence or presence of 1 μM nifedipine prior to collection of whole cell lysates. Lysates were analyzed by Western blotting using antibodies to c-Fos or c-Jun. Lysates were probed with c-Jun antibody were run on a low cross-linking SDS-polyacrylamide gel to separate c-Jun from phosphorylated c-Jun. The retarded electrophoretic mobility of c-Jun protein following buserelin treatment corresponds to a phosphorylation of c-Jun.

GnRH results in increased mRNA for the immediate early genes c-fos and c-jun (14). To examine for effects of VGCC calcium on downstream targets of GnRH-induced MAPKs, αT3-1 lysates were examined for amounts of c-fos and c-jun protein following 1 or 2 h of hormone stimulation in the absence or presence of nifedipine. Buserelin-induced increases in c-Fos protein were reduced in the nifedipine-treated cells, while c-Jun protein amounts and phosphorylation, as measured by retarded electrophoretic mobility shift, were similar in control and nifedipine-treated cells (Fig. 1D).
Cellular Calcium through L-type VGCCs—The ability of nifedipine to block buserelin-stimulated ERK activation suggests that calcium influx through VGCCs is a necessary component of this signaling pathway. To further confirm a role for extracellular calcium, αT3-1 cells were placed in a calcium-free extracellular solution in which the calcium ions had been replaced by magnesium or barium ions to maintain isosmotic conditions immediately prior to stimulation with hormone. The ability of buserelin to activate ERK was completely abolished in the solutions that had magnesium or barium substituted for calcium (Fig. 2A).

It has been shown previously that αT3-1 cells have both L-type and T-type VGCCs (1). To confirm a specific role of L-type channels, a series of experiments was completed to examine the effects of non-L-type calcium influx by administration of nickel, a specific blocker of T-type calcium channels (19). Buserelin activation of ERK in cells that had been treated with nickel was similar to ERK activation observed in control cells (Fig. 2B). These data suggest that buserelin-induced ERK activation specifically required calcium entry through L-type channels.

Stimulation of VGCCs with Bay-K 8644 Activates ERK and c-Fos but Not JNK and c-Jun—To examine the requirement for calcium influx through VGCCs in the signaling pathway coupling the GnRH receptor to ERK activation in more detail, αT3-1 cells were stimulated with the L-type VGCC agonist, Bay-K 8644. Initial studies examining indo-1/AM fluorescence indicated that a prolonged increase in cytoplasmic calcium is observed following treatment of αT3-1 cells with Bay-K 8644 (Fig. 3A). Cells exposed to Bay-K 8644 exhibited an increase in ERK activation similar to that seen when cells were treated for the same time periods with buserelin (Fig. 3B). Bay-K 8644 did not induce activation of JNK. When αT3-1 lysates were examined for increases in c-Fos and c-Jun protein amounts, it was observed that Bay-K 8644 treatment resulted in an induction of c-Fos protein similar to that seen in buserelin-treated cells, while there was no apparent influence on c-Jun protein amounts or activation state (Fig. 3C). These results indicate that calcium influx through L-type VGCCs is a sufficient stimulus for activation of ERK and subsequent c-Fos induction.
no observed increase in calcium signal (Fig. 4B). Cells receiving control solution responded to Bay-K 8644 with a prolonged increase in fluorescence (Fig. 4B). When cells in 35 mM potassium were treated with nifedipine and then buserelin, a transient increase in fluorescence was observed (Fig. 4C). This is probably due to a buserelin-induced release of calcium from internal stores. Voltage-stimulated activation of VGCCs by depolarization of αT3-1 cells treated with 35 mM potassium and then 1 μM nifedipine. The arrow indicates the base line, and the dotted line indicates the approximate base line expected following dilution with 800 μl of high potassium solution (see Experimental Procedures). B, indo-1/AM fluorescence of αT3-1 cells treated with control solution and then 1 μM Bay-K 8644. The arrow indicates the original base line, and the dotted line indicates the new base line following dilution with 800 μl of control solution. C, indo-1/AM fluorescence of αT3-1 cells treated with 35 mM potassium, 1 μM nifedipine, and 10 mM buserelin. The arrow indicates the original base line, and the dotted line indicates the approximate base line expected following the addition of 800 μl of high potassium solution. D, αT3-1 cells were treated for 0, 5, 15, or 30 min with either 1 μM Bay-K 8644 or 35 mM potassium in solutions identical to those used in fluorescence studies. Whole cell lysates were collected and analyzed by Western blotting for phospho-ERK (p-ERK). The blot was then stripped and reprobed with ERK antibody, demonstrating equivalent protein amounts in each lane.

IP₃-released intracellular calcium in this signaling pathway, cells were treated with thapsigargin, an inhibitor of the calcium ATPase that pumps calcium into intracellular stores. Thapsigargin is known to increase cytoplasmic calcium levels and eventually cause depletion of intracellular stores. Initially, the indo-1/AM fluorescence of cells treated with thapsigargin was examined. Thapsigargin stimulated an increase in fluorescence that gradually decreased, presumably as intracellular stores were depleted (Fig. 5A). It is of interest to note that the effect of thapsigargin treatment on cytosolic calcium has a fluorescence profile similar to that seen following Bay-K 8644 stimulation (Fig. 3A). An examination of indo-1/AM fluorescence observed upon buserelin stimulation following thapsigargin treatment suggests that buserelin stimulates a transient increase in cytoplasmic calcium (Fig. 5A, arrow). The buserelin-stimulated indo-1/AM fluorescence in cells pretreated with thapsigargin is greatly reduced in cells that have also been treated with nifedipine (Fig. 5B). These results suggest that the calcium signal activated by buserelin following thapsigargin pretreatment is primarily due to calcium entry through VGCCs.

A series of biochemical studies was then undertaken in which αT3-1 cells were treated with either buserelin or thapsigargin and then examined for ERK activation. Thapsigargin treatment alone did not stimulate ERK phosphorylation (Fig. 5C). It is possible that the calcium signal stimulated by thapsigargin did not activate ERK because it was transient. However, the potassium depolarization experiments indicated that a sustained signal is not always sufficient to stimulate ERK phosphorylation. αT3-1 cells were then stimulated with buserelin in the absence or presence of thapsigargin and examined...
for activation of ERK. Previous studies by others have demonstrated that a similar treatment with thapsigargin blocks IP$_3$-induced intracellular calcium oscillations and buserelin-induced secretion in primary gonadotropes or αT3-1 cells (3, 22, 23). Buserelin-induced ERK activation was only slightly reduced in thapsigargin-treated cells when compared with control cells. Certainly, the effects of thapsigargin are much less than the dramatic reduction in buserelin-induced ERK activation seen following treatment with nifedipine. Taken together, these results suggest that treatment with thapsigargin is sufficient to reduce intracellular calcium stores and that buserelin-induced IP$_3$-mediated increases in intracellular calcium are not required for ERK activation by buserelin.

**Nifedipine Blocks Activation of Raf Kinase by Buserelin**—Raf kinase activation, measured by electrophoretic mobility shift or kinase assay, was examined in cells stimulated with buserelin in the absence or presence of nifedipine (Fig. 6A). Treatment of αT3-1 cells with buserelin for 15 or 30 min resulted in a retarded electrophoretic mobility of Raf protein. The shift to a higher apparent molecular weight has been shown to reflect phosphorylation and thus activation (24). Pretreatment with nifedipine abolished the buserelin-stimulated retardation in Raf electrophoretic mobility, suggesting that VGCC calcium is acting upstream of Raf in the signaling pathway linking the GnRH-R to ERK. As further evidence for nifedipine-sensitive activation of Raf by buserelin, we directly examined Raf kinase activity. Consistent with electrophoretic mobility shift studies, treatment of αT3-1 cells with nifedipine was effective at blocking Raf kinase activity as measured following Raf IP and kinase assay (Fig. 6A). This is the first report of buserelin-induced activation of Raf kinase. Interestingly, treatment of αT3-1 cells with Bay-K 8644 for 15 or 30 min was also sufficient stimulus for inducing hyperphosphorylation of Raf (Fig. 6B).

Additional studies examined the ability of nifedipine to block transcriptional activation of the ERK substrate Elk 1 by an expression vector for a constitutively active form of Raf kinase (Raf-CAAX). αT3-1 cells were transiently transfected by electroporation with an expression vector for Gal4-Elk 1 and a luciferase reporter gene containing five Gal4 binding sites (Fig. 6C). Cotransfection of Raf-CAAX expression vector with Gal4-Elk 1 resulted in a marked increase in transcriptional activation. Administration of the specific MAPK/ERK kinase 1/2 inhibitor, PD98059, blocked Raf-induced Elk 1 activation. In contrast, pretreatment of transfected cells with nifedipine did not reduce Gal4-Elk 1 activation, suggesting that nifedipine administration does not interfere with signaling mechanisms downstream of Raf kinase.

**PKC, Calcium, and MAPK Activation in αT3-1 Cells**—Stimulation of PKC with the phorbol ester PMA has been shown to be sufficient for activation of ERK and JNK in αT3-1 cells. To determine whether VGCC calcium was required for PMA-induced ERK and JNK activation, αT3-1 cells were stimulated with 10 μM PMA for 15 min in the absence or presence of nifedipine. As was seen for buserelin-induced ERK and JNK activation, blockade of L-type VGCCs interfered with the ability of PMA to induce ERK but not JNK (Fig. 7A). Because JNK was still activated in the PMA-stimulated cell treated with nifedipine, it is likely that nifedipine treatment does not directly interfere with the activation of PMA-sensitive PKC isoforms.

Buserelin-induced ERK activation requires PKA-PKC isoforms (10). Because PKA induction of PKC required nifedipine-sensitive calcium, studies were completed to further examine the relationship between buserelin-induced PKC, calcium signals, and ERK activation. Treatment with the protein kinase inhibitor H-89 (25) demonstrated that the ability of buserelin to induce ERK is decreased following treatment with a high dose (10 μM) of H-89 known to inhibit PKC (Fig. 7B) but not the dose (100 nM) specific for protein kinase A inhibition (data not shown). Further, treatment with 30 μM Rp-cAMPS, a specific protein kinase A inhibitor, had no effect on GnRH-induced ERK activation (data not shown). Results from additional biochemical experiments using staurosporine (0.5 μM), GF 109203X (1 μM), and PKC down-regulation by chronic treatment (20 h, 100 nm) also inhibited buserelin-induced ERK activation (data not shown). In a control series of fluorescence studies, we confirmed that a PMA-stimulated increase in intracellular calcium was inhibited by 10 μM H-89 but not 100 nM H-89 (data not shown). H-89 was used in these experiments, since other PKC inhibitors used to inhibit ERK activity in biochemical studies were not useful in parallel fluorescence studies due to nonspecific effects on the fluorescent dye used (data not shown). Studies examining indo-1/AM fluorescence of αT3-1 cells pretreated with control vehicle or H-89 confirmed that the VGCC portion of the buserelin-induced calcium signal is inhibited by 10 mM H-89 (Fig. 7, C and D) but not 100 nM H-89 (data not shown). It is of interest to note that although buserelin was unable to induce the VGCC calcium signal fol-
nM buserelin and 1
rently, GnRH enhances calcium influx through VGCCs in the
that is not influenced by short term removal of extracellular
m

It generates an IP3-mediated increase in intracellular calcium



FIG. 7. PKC is required for buserelin-stimulated but not Bay-K 8644-stimulated ERK activation. A, αT3-1 cells received 10 nM PMA for 0 or 15 min in the absence or presence of nifedipine prior to collect-
ing whole cell lysates for examination of ERK activation by Western blotting using an antibody for phospho-ERK (p-ERK) or JNK activity by kinase assay. The ERK blot was then stripped and reprobed with ERK antibody, demonstrating equivalent protein amounts in each lane. B, αT3-1 cells received 15-min pretreatment with control vehicle or 10 μM H-89 to inhibit PKC activity and were then treated for 0, 15, or 30 min with buserelin in the continued presence of control vehicle or H-89. Whole cell lysates were collected for examination of ERK activation by Western blotting using an antibody for phospho-ERK. The blot was then stripped and reprobed with ERK antibody demonstrating equivalent protein amounts in each lane. C, indo-1/AM fluorescence of αT3-1 cells exposed to 10 nM buserelin. D, indo-1/AM fluorescence of αT3-1 cells pretreated for 15 min with 10 μM H-89 prior to treatment with 10 μM buserelin and 1 μM Bay-K 8644. E, cells received a 15-min pretreatment with control vehicle or 10 μM H-89 to inhibit PKC activity and were then treated with 0, 15, or 30 min of 1 μM Bay-K 8644 in the continued presence of control vehicle or H-89. Whole cell lysates were collected for examination of ERK activation by Western blotting using an antibody for phospho-ERK. The blot was then stripped and reprobed with ERK antibody demonstrating equal protein amounts in each lane.

Following pretreatment with the higher dose of H-89, stimulation with Bay-K 8644 resulted in a robust increase in indo-1/AM fluorescence (Fig. 7D). Therefore, it is unlikely that the higher dose of H-89 has a nonspecific inhibitory effect on L-type calcium channels. Further, Bay-K 8644 stimulation of αT3-1 cells results in activation of ERK in the absence or presence of H-89 treatment (Fig. 7E). These data support the conclusion that PKC may act upstream of the L-type channel in the buserelin signaling pathway leading to ERK activation.

DISCUSSION

GnRH has multiple actions on pituitary gonadotropes including the control of gonadotropin hormone biosynthesis and se-
cretion. Previous studies have suggested a role for calcium in
GnRH-induced activation of MAPKs and IEGs (11, 15). How-
ever, specific contributions of the discrete pools of calcium
activation by GnRH and the mechanisms involved in regulation
were unclear. GnRH activates two separate and independent
calci um signals in αT3-1 cells and primary gonadotropes (1–3).
It generates an IP3-mediated increase in intracellular calcium
that is not influenced by short term removal of extracellular
calcium or pharmacological blockade of VGCCs (1, 3). Concur-
rently, GnRH enhances calcium influx through VGCCs in the
plasma membrane, possibly via a PKC-dependent mechanism
(26). An initial study using rat pituitary cells in primary cul-
ture indicated that activation of ERK by the GnRH agonist
buserelin could be inhibited by treatment with the L-type cal-
cium channel blocker nifedipine, providing evidence for the
fidelity of the αT3-1 cell model system. Studies described here
made use of the αT3-1 cell model for biochemical and biophys-
ical experiments to explore the potential contributions of the
two GnRH-regulated calcium signals in the activation of
MAPKs and specific IEG targets.

Buserelin did not induce ERK phosphorylation in cells main-
tained in calcium-free media or treated with the L-type calcium
channel blocker, nifedipine. ERK was still phosphorylated in
cells treated with nickel to block T-type calcium channels. In
contrast to the effects observed for ERK phosphorylation, nife-
dipine treatment of αT3-1 cells had no effect on buserelin-
stimulated JNK activity, suggesting that there is no require-
ment for a VGCC signal. The buserelin-mediated increase in
c-Fos protein was abolished in nifedipine-treated cells, while
c-Jun induction and activation were unaffected. ERK is the
primary activator of c-Fos, while JNK activity is sufficient for
c-Jun activation, consistent with the reports of others (27).
The studies presented here provide novel evidence in support of
an absolute requirement for calcium influx through L-type chan-
nels in the activation of the ERK (but not JNK) cascade by
buserelin.

Treatment with thapsigargin produces a transient calcium
signal, most likely due to the release of calcium from IP3-
sensitive stores, and was not sufficient for activation of ERK
and induction of c-Fos. Buserelin and Bay-K 8644 induced a
sustained calcium signal; therefore, one could suggest that it is
the temporal and not spatial nature of the calcium signal that
is elemental in ERK activation. While we cannot completely
rule out the necessity for a sustained VGCC calcium signal, our
data do not support this conclusion. A prolonged increase in
cytoplasmic calcium induced by elevated potassium was not a
sufficient stimulus for activation of ERK. Further, data pre-

tened in Fig. 5A identify by indo-1/AM fluorescence the spe-
cific calcium signal that is sufficient for buserelin-induced ERK
activation. This transient calcium signal is present in cells that
have been treated with thapsigargin yet is blocked in cells
pretreated with nifedipine (Fig. 5B), suggesting that it is com-
oposed of calcium influx through VGCCs. It is of interest to note
that the similar transient calcium signal seen following treat-
ment with nifedipine (Fig. 1A) that is presumably due to re-
lease of calcium from internal stores is not sufficient for ERK
activation, while a transient signal resulting from extracellular
calcium influx (Fig. 5A) permits activation of ERK. These data
strengthen our hypothesis that there is an absolute require-
ment for calcium influx through VGCCs for GnRH-induced
ERK activity.

It is not completely clear why activation of VGCCs by depo-
larization with high KCl is not a sufficient stimulus for activa-
tion of ERK. This finding indicates that VGCC activity stimu-
lated solely by membrane potential changes differs from VGCC
activity stimulated by Bay-K 8644 or buserelin. However, the
magnitude of a VGCC calcium signal can differ depending on
the stimulus for channel activation. Activation of VGCCs with
Bay-K 8644 increases the time that the channels are in the
open state when compared with channel activation by depolar-
ization, thereby increasing the flux of calcium through the
channel (28, 29). Similar results have been reported in studies
examining the effects of VGCC phosphorylation on channel
activity (30, 31). This could explain why stimulation of VGCCs
with buserelin or acute PMA treatment is a sufficient stimulus
for ERK activation, while depolarization of cells with elevated

L-type VGCC Stimulation Activates ERK
potassium is not. It has been suggested recently that cells may possess defined localized regions, or microdomains, of high calcium following electrical stimulation (32). These microdomains occur at the mouth of VGCC such that very high (>100 μM) calcium levels are reached for very brief periods of time (milliseconds) during channel opening. Such a localized increase in calcium is great in magnitude but brief in duration could serve as an important trigger for cellular signal transduction. It could allow for activation of a calcium-sensitive protein that is temporally and spatially restricted, thereby preventing exposure of the global cellular environment to levels of calcium that are potentially cytotoxic. Microdomains of calcium have been described with regard to regulation of secretion in presynaptic nerve terminals where entry of calcium through VGCCs is the signal for exocytosis. It is likely that similar mechanisms could exist in other secretory cells for regulation of specific enzyme cascades that contribute to the regulation of gene transcription. Experiments that provide definitive results in regard to the presence and function of a microdomain of calcium would be technically difficult. We have used a multidisciplinary approach to begin to examine whether a specific calcium signal can influence a selective target in a complex cellular system. Further biophysical studies are required to analyze calcium flux through VGCCs following activation by various stimuli. Regardless, it is clear that activation of the signal transduction cascade leading to phosphorylation of ERK requires a very specific signal involving activation of L-type VGCCs.

The influence of calcium influx through VGCCs on elements of the GnRH signaling pathway downstream of PKC was examined. PMA-induced ERK was blocked by nifedipine, while PMA-stimulated JNK was unaffected. Activation of Raf kinase by buserelin was inhibited by nifedipine. However, nifedipine did not block downstream elements of the ERK pathway when activation was at the level of Raf kinase. These data suggest that VGCC calcium is acting downstream of PKC but upstream of Raf. Previous reports have indicated a sensitivity of buserelin-activated ERK to the tyrosine kinase inhibitor genistein (11). Therefore, it is conceivable that a tyrosine kinase may exist in the pathway downstream of PKC but upstream of Raf. Of interest, treatment with thapsigargin. These results are consistent mechanistically with our studies. Bay-K 8644 was a sufficient stimulus for α-subunit gene transcription (18). Our data are in agreement with these studies and provide a potential mechanism, namely activation of ERK, by which calcium flux through L-type channels may be influencing α-subunit gene transcription. Recent data from experiments using GH3 cells overexpressing rat GnRH receptors (GGH(3)-1' cells) suggest that modulation of the α-subunit gene and the luteinizing hormone β-subunit and follicle-stimulating hormone β-subunit genes may have differential sensitivities to influx of extracellular calcium through L-type VGCCs (33). Consistent with our hypothesis that VGCC calcium and ERK are crucial for GnRH-induced α-subunit transcription in αT3-1 cells and possibly primary gonadotropes, GnRH-mediated stimulation of α-subunit in GGH(3)-1' cells could be blocked by L-type calcium channel blockers. Interestingly, transcriptional activation of luteinizing hormone β-subunits and follicle-stimulating hormone β-subunits was not sensitive to block or activation of VGCCs (33). These studies have interesting implications for differential use of signal transduction pathways by GnRH for regulation of multiple MAPKs and modulation of gonadotropin subunit gene expression.

The strict requirement for calcium influx in buserelin-induced ERK and Fos activation as well as the ability of a VGCC signal to activate ERK and Fos differ from the paradigms that have been observed for other receptors coupled to phospholipase C activation. Angiotensin II receptors are coupled to Goq/11 and when bound to agonist can induce an IP3-mediated calcium signal as well as a VGCC signal (34). However, the influence of discrete calcium signals upon angiotensin II-induced MAPK activation differs among cell types. Experiments in adrenal glomerulosa cells have indicated that activation of a signal transduction pathway that includes Raf, ERK, and Fos does not require either the VGCC or IP3-mediated calcium signal (35). In contrast, in smooth muscle cells, angiotensin II-stimulated MAPK activity has been shown to be dependent upon IP3-released calcium but not calcium influx through VGCCs (36). Studies of Goq-coupled endothelin B receptors in smooth muscle cells have demonstrated that VGCC calcium is

![Diagram](image-url)
required for Raf and ERK activation; however, calcium influx alone is not a sufficient signal for MAPK activation (37). The GnRH receptor is unique when compared with other heterotrigenic G protein-coupled receptors in that it lacks the C-terminal tail found in other members of this receptor superfamily. Interestingly, it is the endothelin B receptor cytoplasmic C-terminal tail that has been shown to be required for ERK activation and increased cytosolic calcium (38). The distinct signaling mechanisms utilized by GnRH may be related to structural differences in the receptor.

Taken together, the results of the current studies suggest that signaling pathways leading to activation of MAPKs and control of immediate early genes may have differential sensitivities to specific fluxes of cell calcium. There is a striking divergence in the GnRH-induced signaling pathways leading to activation of ERK and JNK in regard to requirement and sufficiency of a VGCC calcium signal. Further, our evidence suggests that some component of the signaling pathway leading to ERK activation can discriminate between concurrently activated calcium signals. This has interesting implications for control of gene transcription in many cell types. Further research is necessary to determine what, in the cascade of signaling events leading to ERK activation, confers sensitivity to a concise, spatially restricted calcium signal. Based on our results and those of others, we propose a working hypothesis in which influx of extracellular calcium through L-type calcium channels in the plasma membrane is required for activation of ERK by buserelin in αT3-1 cells (Fig. 8). The influence of calcium appears to be upstream of the ERK kinase, MAPK/ERK kinase 1/2, and Raf in cells stimulated with buserelin. We suggest that GnRH-stimulated PKC may function to modulate L-type calcium channels, possibly through phosphorylation, as has been suggested (39). We found no evidence of a requirement for calcium coming from IP3-released stores in ERK activation and increased cytosolic calcium (38). The distinct signaling mechanisms utilized by GnRH may be related to structural differences in the receptor.

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