Dynamin Is Required for GnRH Signaling to L-Type Calcium Channels and Activation of ERK

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We have shown that GnRH-mediated engagement of the cytoskeleton induces cell movement and is necessary for ERK activation. It also has previously been established that a dominant negative form of the mechano-GTPase dynamin (K44A) attenuates GnRH activation of ERK. At present, it is not clear at what level these cellular events might be linked. To explore this, we used live cell imaging in the gonadotrope-derived /H9251T3–1 cell line to determine that dynamin-green fluorescent protein accumulated in GnRH-induced lamellipodia and plasma membrane protrusions. Coincident with translocation of dynamin-green fluorescent protein to the plasma membrane, we demonstrated that dynamin co-localizes with the actin cytoskeleton and the actin binding protein, cortactin at the leading edge of the plasma membrane. We next wanted to assess the physiological significance of these findings by inhibiting dynamin GTPase activity using dynasore. We find that dynasore suppresses activation of ERK, but not c-Jun N-terminal kinase, after exposure to GnRH agonist. Furthermore, exposure of /H9251T3–1 cells to dynasore inhibited GnRH-induced cyto-architectural rearrangements. Recently it has been discovered that GnRH induced Ca\(^{2+}\) influx via the L-type Ca\(^{2+}\) channels requires an intact cytoskeleton to mediate ERK phosphorylation. Interestingly, not only does dynasore attenuate GnRH-mediated actin reorganization, it also suppresses Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels visualized in living cells using total internal reflection fluorescence microscopy. Collectively, our data suggest that GnRH-induced membrane remodeling events are mediated in part by the association of dynamin and cortactin engaging the actin cytoskeleton, which then regulates Ca\(^{2+}\) influx via L-type channels to facilitate ERK phosphorylation. (Endocrinology 157: 831–843, 2016)

The oscillatory discharge of GnRH from hypothalamic neurons is obligatory for synthesis and secretion of the gonadotropins, LH and FSH (1–4). The gonadotropins are heterodimeric glycoproteins consisting of a common α-subunit and a unique hormone-specific β-subunit that once released into systemic circulation, regulate gonadal development and function by stimulating spermatogenesis, folliculogenesis, and ovulation. Given the essential role of GnRH in maintaining fertility, much effort has gone into identifying the signaling mechanisms used by the GnRH receptor (GnRHR).

After GnRH activation, the GnRHR predominantly couples to G\(_{\alpha_{q/11}}\), resulting in activation of phospholipase C. This leads to cleavage of phoshatidylinositol-4,5-bisphosphate, to generate inositol-1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol, and subsequent activation of protein kinase C. These intracellular messengers bind to and activate the GnRHR, which then transduces signals through the Ras-Raf-MAP kinase (ERK) cascade, culminating in cell division and differentiation (1–4). The oscillatory discharge of GnRH from hypothalamic neurons is obligatory for synthesis and secretion of the gonadotropins, LH and FSH (1–4). The gonadotropins are heterodimeric glycoproteins consisting of a common α-subunit and a unique hormone-specific β-subunit that once released into systemic circulation, regulate gonadal development and function by stimulating spermatogenesis, folliculogenesis, and ovulation. Given the essential role of GnRH in maintaining fertility, much effort has gone into identifying the signaling mechanisms used by the GnRH receptor (GnRHR).

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kinase C (PKC) isozymes (1, 5). Additionally, GnRHR activation leads to increased cytoplasmic Ca$^{2+}$ mobilization in both αT3-1 cells and primary pituitary gonadotropes (2, 3). IP$_3$ facilitates Ca$^{2+}$ mobilization from the endoplasmic reticulum via activation of the IP$_3$ receptors, whereas PKC mobilizes extracellular Ca$^{2+}$ influx through activation of voltage-gated L-type Ca$^{2+}$ channels (VGCCs) (4). GnRH-mediated Ca$^{2+}$ mobilization from both sources is essential for activation of multiple MAPK pathways in gonadotropes, including ERK, c-Jun N-terminal kinase (JNK), and p38-MAPK. Release of Ca$^{2+}$ from the endoplasmic reticulum facilitates JNK activation, whereas extracellular Ca$^{2+}$ influx through VGCCs facilitates ERK activation (6). The ERK signaling module has been studied extensively in gonadotropes and is linked to the transcriptional regulation of the LHβ gene through induction of the immediate early gene Egr-1 (7–9). Female mice deficient in either in Egr-1 or ERK1/2 are infertile due to deficiencies in LHβ production (10, 11). Past work from our group has also shown that disruption of the actin cytoskeleton inhibits GnRH-induced activation of ERK (12). Presently, the biochemical link by which GnRHR activation causes both actin reorganization and ERK activation remains unclear.

The actin cytoskeleton is involved in important cellular functions underlying cell movement, shape, and intracellular and transmembrane protein trafficking (13, 14). Previous work from our group and others has shown that gonadotropes rapidly (within 60 s) induce actin polymerization to form membrane ruffles, lamellipodia, and filopodia in the presence of GnRH (12, 15). Recently, it has been found that GnRH induces actin remodeling events through the activation of cortactin, which facilitates its association with actin-related protein (Arp) 2/3 complex to induce actin branching and remodeling (16). Not only does cortactin localize to areas of dynamic actin-containing structures, it also directly binds to dynamin via its C-terminal SH3-domain in these areas (17–19).

Dynamin is a large GTPase and proline-rich domain (PRD)-containing protein that possesses mechanochemical properties important in membrane remodeling events and fission (20). There are 3 different dynamin isoforms that are selectively expressed in cells. Dynamin I is specifically expressed in neuronal cells (21), dynamin II is ubiquitously expressed (22), and dynamin III is restricted to the testis (23). Regardless of the isoform, dynamin appears to be associated with remodeling of the actin cytoskeleton (24). Overexpression of the dominant negative dynamin-K44A mutant, impaired in hydrolyzing GTP, perturbs many F-actin-rich cellular structures (25–27). Consistently, αT3–1 cells transfected with dynamin-K44A resulted in a loss of not only GnRH-induced actin remodeling events (data not shown), but also GnRH-mediated ERK activation (28). Taken together, we suggest that GnRH-induced gonadotrope plasticity may be modulated through the interaction of dynamin and cortactin to effectively engage the actin cytoskeleton to facilitate ERK activation.

Our studies provide evidence that GnRHa activation of αT3–1 gonadotropes leads to redistribution of dynamin to the leading edge of gonadotropes where it colocalizes with the actin cytoskeleton and cortactin to facilitate actin assembly events. Inhibition of dynamin GTPase activity, via dynasore, results in suppression of actin reorganization and also phosphorylated ERK. Consistent with the loss of actin reorganization, dynasore also decreases Ca$^{2+}$ influx through VGCCs in response to GnRH, which consequently leads to a significant reduction in ERK phosphorylation. Taken together, we demonstrate that after GnRHR activation, dynamin associates with cortactin to effectively engage the actin cytoskeleton. Reorganization of actin is critical for extracellular Ca$^{2+}$ influx through L-type channels and subsequently ERK activation necessary for LHβ synthesis.

**Materials and Methods**

**Materials**

The antiphospho-ERK1/2 (p-ERK), antirabbit horseradish peroxidase (HRP), antimouse-HRP antibodies, and protein A/G agarose beads were purchased from Santa Cruz Biotechnology, Inc. The Dyngo 4a inhibitor and the anticortactin and antidymin I/II antibodies were purchased from Abcam. The anti-ERK1/2 (ERK1/2), antiphospho-JNK 1/2 (p-JNK), and anticortactin were purchased from Cell Signaling Technology. The anti-LHβ antibody was purchased from the National Hormone and Peptide Program (National Institute of Diabetes and Digestive and Kidney Diseases). Antidynamin I/II antibody and Matrigel was purchased from BD Biosciences. All fluorescently labeled Alexa Fluor secondary antibodies were purchased from Molecular Probes-Life Technologies. The dynamin I and II primers were purchased from Integrated DNA Technologies. GnRH, Buserelin (GnRHa), dynasore, and the anti-β-tubulin were purchased from Sigma. Glass bottom microwell dishes for confocal studies were obtained through MatTek. Phorbol 12-myristate 13-acetate (PMA) was purchased from Fisher Scientific. The dynamin II-green fluorescent protein (GFP) was a generous gift from Dr Gary Whittaker (Cornell University, Ithaca, NY).

**Cell culture**

αT3–1 or LβT2 cells (a generous gift from Dr Pamela Mellon, University California, San Diego) were maintained in high glucose DMEM containing 2mM glutamine, 100-U penicillin/mL, 100-µg streptomycin/mL, 10% fetal bovine serum by Life Technologies. All cells were grown in 5% CO$_2$ at 37°C in a humidified environment.
**Ovine pituitary culture**

White-faced Colorado ewes of similar age (2–4 y) and weight were maintained under natural conditions at the Animal Reproduction and Biotechnology Laboratory Sheeps Facility, Fort Collins, CO. A follicular phase ewe was killed with an overdose of sodium pentobarbital, and the pituitary gland was removed aseptically. The pituitary was rinsed free of blood and cells dispersed enzymatically using collagenase, hyaluronidase, and deoxyribonuclease at 37°C for 90 minutes as previously described (29). Dissociated cells were then suspended in culture medium (DMEM supplemented with 10% horse serum [Gemini Bio-Products, Inc], 2.5% fetal bovine serum, 1% nonessential amino acids, 100-IU/mL penicillin, and 100-μg/mL streptomycin). Cells (5 × 10^5) in 2-mL media were plated in glass-bottom microwell dishes and cultured at 37°C in a humidified atmosphere of 5% CO₂. All procedures involving animals were approved by the Colorado State University Animal Care and Use Committee and complied with National Institutes of Health guidelines (protocol number 13-4349A).

**RNA extraction, reverse transcription, PCR, and quantitative RT-PCR**

Total RNA was extracted from αT3–1 cells using E.Z.N.A. Total RNA kit (Omega) according to the manufacturer’s instructions. To obtain single-stranded cDNA, 1.0 μg of total RNA were reverse transcribed using iScript Reverse Transcription SuperMix (Bio-Rad). cDNA was subject to PCR and quantitative polymerase chain reaction analysis with primers specific for the isoform of that captured images every 30 seconds for 20 minutes. Quantitative PCR data were analyzed by the

**Phalloidin staining**

αT3–1 cells were plated in glass-bottom microwell dishes coated with Matrigel (1:100). Selected cells were pretreated with dynasore for 30 minutes and then treated in the presence or absence of either vehicle (0) or GnRHa (10nM) for 10 minutes. Cells were fixed in 4% PFA for 20 minutes, and blocked in PBS containing 3% BSA for 1 hour. Cells were then exposed to an anti-LH antibody (1:250) in PBS containing 3% BSA for 2 hours. For visualization, cells were incubated with Alexa Fluor 594 phalloidin diluted in 200-μL 1% BSA/PBS and applied to the cells for 20 minutes at room temperature.

Ovine pituitary cells were serum starved for 2 hours and then administered vehicle or dynasore (80μM) for 30 minutes. After pretreatment, either vehicle or 10nM GnRHa was administered or 10 minutes. Cells were washed with cold PBS and fixed in 4% PFA for 30 minutes, permeabilized with 0.5% Triton X-100 in PBS for 20 minutes, and blocked in PBS containing 3% BSA for 1 hour. Cells were preincubation to an anti-LHβ antibody (1:250) in PBS containing 3% BSA for 2 hours. For visualization, cells were incubated with Alexa Fluor 594 phalloidin diluted by 4′,6-diamidino-2-phenylindole (DAPI) and imaged by CLSM.

**Live cell confocal microscopy**

Live cell confocal microscopy was conducted using either a Zeiss LSM510 or LSM710 confocal laser-scanning microscope (CLSM) under a ×63 oil objective with appropriate fluorescent filters. αT3–1 cells (5 × 10^5) were transiently transfected with dynamin-GFP using SuperFect reagent (QIAGEN) for 24 hours were grown on glass bottom microwell dishes coated with Matrigel (1:100 dilution). Cells were serum starved for 2–4 hours before treatment. Selected fluorescent cells were imaged before the administration of GnRHa. After the addition of 10nM GnRHa, the selected cell was imaged using a time series function that captured images every 30 seconds for 20 minutes.

**Immunocytochemistry**

αT3–1 cells were grown in a 35-mm dish with a glass bottom coverslip coated with Matrigel (1:100) and serum starved for 2–4 hours. Cells were then treated with either vehicle (0) or GnRHa 10nM. After treatment, cells were washed twice with 0.01M PBS and then fixed by submersion in 4% paraformaldehyde (PFA) in PBS for 30 minutes at room temperature. The cells were then rinsed twice with PBS and permeabilized for 20 minutes in 0.5% Triton X-100/PBS. After permeabilization, cells were rinsed twice with PBS and blocked in 3% BSA/PBS for 1 hour at room temperature. Cells were incubated with an anti-cortactin antibody followed by Alexa Fluor 488-conjugated secondary antibody. Cells were washed and reblocked before the administration of dynamin antibody. Dynamin was visualized using an Alexa Fluor 594-conjugated secondary antibody and imaged using CLSM. For visualization of dynamin and actin, cells were treated and stained for dynamin as described above.

Actin was stained using Alexa Fluor 594 phalloidin diluted in 200-μL 1% BSA/PBS and applied to the cells for 20 minutes at room temperature.

**Western blotting**

αT3–1 or LβT2 cells (2 × 10^6) were grown overnight in a 6-well culture dish and then serum starved for 2–4 hours. Cells were then washed twice in PBS, lysed in radio-immunoprecipitation assay (RIPA) buffer and subjected to SDS-PAGE (acylamide:bis-acylamide ratio of 29:1) and electrophoiled to polyvinylidene difluoride membranes. Membranes were blocked in Tris-buffered saline with Tween 20 (TBST)/2% casein. Membranes were then incubated for 1 hour with either an anti-p-ERK antibody (1:1000) or overnight with an anti-p-JNK antibody (1:2000). Blots were washed (3 × 10 min) with TBST and then incubated with a 1:10 000 dilution of an appropriate HRP-conjugated secondary antibody for 1 hour at room temperature. All membranes were washed for 30 minutes (3 × 10

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min) with TBST after secondary antibody and then visualized by chemiluminescence using Pierce SuperSignal reagents, Bio-Rad ChemiDoc XR5+ and Image Lab Software. After developing, blots were then stripped at room temperature with 100mM 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), and 62.5mM Tris-HCl (pH 6.7) heated to 50°C for 20 minutes. After stripping, membranes were washed twice for 15 minutes with TBS and blocked with 2× casein for 1 hour. Blots were then reprobed with a 1:1000 dilution of either an anti-β-tubulin antibody (1:5000), an anti-ERK1/2 (1:5000) antibody. After washing in TBST, blots were incubated with a 1:5000 dilution of the appropriate HRP secondary antibody and then imaged as described above.

**Immunoprecipitations**

The αT3–1 cells (2 × 10^7) were grown in 100-mm tissue culture dishes overnight. The next day, cells were serum starved for 2–4 hours and treated in the absence or presence of 10nM GnRHa for 15 minutes and lysed in RIPA buffer. Lysates were precleared and then incubated with anticortactin antibody (mouse) or nonimmune mouse serum for 2 hours at 4°C. Protein A/G agarose beads (30 µL) were added, and the samples were rocked overnight at 4°C. Beads were then washed 3 times in PBS, resuspended in 40 µL of 1× SDS loading buffer, and boiled for 5 minutes. Immunoblots were performed using an antidymanin antibody. For loading controls, the membrane was stripped and probed using an anticortactin antibody.

**Electrophysiology and total internal reflection fluorescence microscopy**

αT3–1 cells were plated onto Matrigel-coated glass-bottomed dishes (1:100) 24 hours before experimentation and Ca^{2+} was imaged at previously described (30). Simultaneous electrophysiology and Ca^{2+} imaging experiments were carried out using the conventional dialyzed whole-cell clamp technique as described previously (31–34). Briefly, Ca^{2+} influx through L-type Ca^{2+} channels was visualized with a TILL Photonics through-the-lens total internal reflection fluorescence (TIRF) system built around an inverted Olympus IX-71 microscope with a 100X TIRF oil-immersion objective (numerical aperture 1.45) and an Andor iXON EMCCD camera (Andor Technology). To monitor Ca^{2+} influx, gonadotropes were loaded with the Ca^{2+} indicator fluo-5F (200µM) and an excess of EGTA (10mM) to lower background noise while minimally interfering with fluo-5F via the patch pipette. The membrane potential was controlled with an Axopatch 200B amplifier (Molecular Devices); fluo-5F excitation was achieved with a 491-nm laser with excitation and emission light being separated with appropriate filters. Ca^{2+} influx was recorded with 2mM external Ca^{2+} at a frame rate of 50 Hz and holding potential of ~70 mV to increase the driving force for Ca^{2+} entry. To preclude potential contaminating Ca^{2+} release events from the endoplasmic reticulum, the Ca^{2+}–ATPase inhibitor thapsigargin (1µM) was present during all experiments. αT3–1 cells were pretreated with vehicle or dynasore (80µM) for 10 minutes before acute exposure to GnRH (3nM). Cells were imaged during the pretreatment and for an additional 10 minutes after GnRH treatment. All experiments were performed at room temperature (22°C–25°C).

**L-type Ca^{2+} channel sparklet analysis**

Background-subtracted fluo-5F fluorescence signals were converted to intracellular Ca^{2+} concentrations ([Ca^{2+}]), as described previously (31, 32, 34, 35). Briefly, fluo-5F fluorescence images were analyzed with custom software kindly supplied by L. Fernando Santana (University of Washington, Seattle, WA), and L-type Ca^{2+} channel sparklet activity was determined by calculating the number of quantal levels detected and probability that the site is active (nP) of each site, where n is the number of quantal levels detected, and P is the probability that the site is active. nP values were obtained using pCLAMP version 10.0 (Molecular Devices) on imported [Ca^{2+}], time-course records using an initial unitary [Ca^{2+}], elevation of approximately 20nM as determined empirically. Active L-type Ca^{2+} channel sparklet site densities (Ca^{2+} sparklet sites per µm^2) were calculated by dividing the number of active sites by the area of cell membrane visible in the TIRF images. Image stacks selected for analysis were obtained between 5 and 10 minutes of pharmacological manipulation.

Normally distributed data are presented as mean ± SEM. Two-sample comparisons of these data were performed using either a paired or unpaired (as appropriate) 2-tailed Student’s t test, and comparisons between more than 2 groups were performed using a one-way ANOVA with Tukey’s multiple comparison post hoc test. L-type Ca^{2+} channel sparklet activity (ie, nP) datasets were bimodally distributed; thus, 2-sample comparisons of nP data were examined with the nonparametric Wilcoxon matched pairs test (2 tailed), and comparisons between more than 2 groups were performed using the nonparametric Friedman test with Dunn’s multiple comparison post hoc test. Arithmetic means of nP datasets are indicated in the figures (solid gray horizontal lines) for nonstatistical visual purposes, and dashed gray lines mark the threshold for high-activity Ca^{2+} sparklet sites (nP ≥ 0.2). Values of P <.05 were considered significant and asterisks used in the figures indicate a significant difference between groups.

**Measurement of intracellular calcium**

αT3–1 cells were plated the day before at 3 × 10^5 in 35-mm glass-bottomed dishes. Cells were loaded at room temperature with fura 2 (5µM) and 0.1% Pluronic F-127 for 30 minutes in fluorescence buffer (FB) (145mM NaCl, 5mM KCl, 1mM Na2HPO4, 0.5mM MgCl2, 1mM CaCl2, 10mM HEPES, and 5mM glucose; pH 7.4). After loading, cells were washed twice in the same buffer and incubated for 30 minutes in FB to allow for ester hydrolysis. Treatment solutions (1-µL Dimethyl sulfoxide (DMSO), as a vehicle control, 80µM dynasore, 0.5µM nicardipine, or both dynasore and nicardipine) were added directly into coverslip dishes before imaging. Cells were mounted on the stage of the inverted fluorescent microscope (Nikon TSM) attached to dual-wavelength Fluorescence Imaging System InCyt2 (Intracellular Imaging) and imaged for 5 minutes before and after the application of 3nM GnRH. Changes in [Ca^{2+}], in individual cells were measured as the ratio of fluorescence at 340/380-nm excitation and 510-nm emission, using an InCyt2 imaging system. The individual responses of 14–18 cells in one dish (14–20 dishes per treatment) were aligned and averaged using the numerical analysis software (CalciumComp) developed by an engineer consultant (K. J. Bois, Fort Collins, CO). Only cells that responded to treatment were used for data analysis. Responses are reported as integrated [Ca^{2+}], increase over baseline for 2 minutes.
Statistical analysis

All statistical analysis was performed using GraphPad prism software. Data are expressed as mean ± SEM of at least 3 independent experiments. Results were analyzed for significance using an unpaired Student’s t test or one-way ANOVA. Post hoc group comparisons were made using Tukey’s honestly significant difference using the critical value P ≤ .05 for declaring significance.

Results

Dynamin II is predominately expressed in gonadotropes and colocalizes with the actin cytoskeleton after GnRH stimulation

To begin addressing the mechanistic link between dynamin, the actin cytoskeleton and MAPK activity, we first assessed the expression of different dynamin isoforms in our gonadotrope derived αT3–1 cell model. In this study, we used PCR and quantitative RT-PCR to examine the relative expression profiles of both dynamin I and II in αT3–1 cells. Dynamin III, expressed predominately in the testis and brain, was not found in our samples (data not shown). We found αT3–1 cells express approximately 35 times more dynamin II mRNA than dynamin I (Figure 1).

Dynamin possesses mechanochemical properties important in membrane remodeling events and fission (20). We have previously found that gonadotrope cell lines and ex vivo gonadotropes display a high degree of plasticity after GnRH treatment due to actin reorganization events (12, 16, 36). To investigate the role that dynamin may play in the initiation of actin remodeling events, we used live cell imaging to visualize dynamin-GFP redistribution after GnRHR activation. αT3–1 cells were transiently transfected with cDNA encoding a dynamin II-GFP fusion protein. Cells were imaged by CLSM before GnRHa treatment (10nM) and at 30 seconds intervals for 10 minutes after GnRHa activation. Upon GnRHa treatment, a portion of dynamin-GFP is redistributed from the cytosol to the plasma membrane in regions indicative of actin remodeling (Figure 2A). We next wanted to confirm that dynamin reorganization was associated with the actin cytoskeleton. After 10 minutes of GnRHa treatment, endogenous dynamin I/II was colocalized with the actin cytoskeleton and a portion is redistributed to the leading edge of αT3–1 (Figure 2B). These results suggest GnRHR activation leads to association of actin and dynamin in areas of cyto-architectural rearrangement.

Figure 1. Dynamin II is predominately expressed in αT3–1 gonadotropes. A, Total RNA was extracted from αT3–1 cells using E.Z.N.A. RNA kit, and dynamin I and II mRNA expression levels were visualized using PCR. B, αT3–1 cells were treated as described in A except dynamin I and II mRNA expression levels were quantified using quantitative RT-PCR. Dynamin I and II mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase and are represented as ΔCt mRNA expression: *** P < .001.

Figure 2. GnRHR stimulation causes dynamin redistribution and colocalization with actin. A, αT3–1 cells grown on glass-bottom microwell dishes were transfected with dynamin-GFP for 24 hours. Cells were treated with 10nM GnRHa and imaged by CLSM every 30 seconds for 20 minutes. Selected panels represent dynamin-GFP expression in the same live cell before GnRHa (0) and 10 minutes after GnRHa treatment. B, αT3–1 cells were grown on glass-bottom microwell dishes and incubated in the presence or absence of 10nM GnRHa for 10 minutes. Cells were fixed in 4% PFA and immunostained for dynamin and visualized using an Alexa Fluor 488 (green) secondary antibody. Cells were then stained with Alexa Fluor 594-conjugated phalloidin (red) and imaged by CLSM. The red box highlights the colocalization of dynamin and actin at a higher resolution.
Inhibition of dynamin GTPase activity suppresses GnRH-mediated activation of ERK but not JNK

Based on our data in the previous section, dynamin is likely to play a role in GnRH-mediated engagement of the cytoskeleton. Interestingly, our previous work also highlights that an intact cytoskeleton is necessary for ERK. At present, it is not clear at what level these cellular events might be linked. To begin exploring these issues, we used dynasore, a cell-permeable pharmacological inhibitor that interferes with GTPase activity of both dynamin I and II (38). αT3–1 cells were pre-treated with either vehicle (0) or increasing doses of dynasore for 30 minutes. ERK phosphorylation was detected 10 minutes after GnRHa treatment. After dynasore treatment, GnRH-mediated ERK phosphorylation remained unchanged at 1μM and 40μM concentrations. However, dynasore treatment was capable of significantly decreasing ERK phosphorylation in a dose-dependent manner (80μM and 160μM) (Figure 4A). The 80μM dose of dynasore is consistent with other previously published work in neuronal cell lines and Chinese hamster ovary cells (39). To confirm the role of dynamin in specifically regulating ERK phosphorylation after GnRH exposure, we also investigated JNK activation. We detected JNK phosphorylation after 10 minutes of GnRHa treatment, which persisted despite exposure to increasing doses of dynasore (Figure 4B). Thus, dynamin appears to be an upstream mediator involved in ERK activation but not that of JNK.

To confirm our results were not an artifact of either cell line or dynamin inhibitor choice, we validated our findings in the gonadotrope derived LβT2 cell line. We also analyzed the effect of an additional inhibitor to dynamin GTPase activity, Dyno 4a (dyngo). αT3–1 or LβT2 cells were pretreated as described above with either dynasore (80μM) or dyngo (30μM). Cells were then treated with 10nM GnRHa for 10 minutes. Dose-response studies revealed that 30μM of dyngo was an effective concentration for blocking dynamin GTPase activity in both cell lines (data not shown). We find that regardless of cell line or dynamin inhibitor, the loss of dynamin GTPase activity leads to a reduction in GnRH induced ERK phosphory-
Inhibition of dynamin GTPase activity blunts actin reorganization in response to GnRH

If our hypothesis is correct that dynamin is involved in actin cytoskeletal reorganization after GnRH, presumably dynasore treatment would inhibit these cytoarchitectural changes. To explore this possibility, we assessed whether inhibition of dynamin would attenuate GnRH-induced actin remodeling in both αT3–1 and primary ovine pituitary cells. αT3–1 and ovine pituitary cells were pretreated with either vehicle or dynasore (80 μM) for 30 minutes and then treated with either 0 or GnRH (10nM) for 10 minutes. Cells were fixed and stained for Alexa Fluor 488 phalloidin and imaged with CLSM. Our results confirm our previous studies that actin remodeling occurs after GnRH stimulation; however, pretreatment with dynasore abrogates GnRH-induced actin reorganization events in both αT3–1 cells (Figure 6A) and primary pituitary cells (Figure 6B). Thus, dynamin is not only found associated with actin binding proteins but is involved in regulating actin dynamics in gonadotropes.

Dynasore decreases Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels after GnRH treatment

It is well known that VGCC activity is necessary for ERK activation. Recent data have highlighted that (VGCC) activity is also dependent on an intact actin cytoskeleton (30). Because inhibition of dynamin blunted actin reorganization and ERK activation, we were intrigued with possibility that dynamin may have an effect on VGCC signaling. To gain a better understanding of the mechanistic actions of dynamin on VGCC activity we assessed channel activity after dynamin inhibition using dynasore. We used electrophysiology combined with TIRF microscopy to visualize subplasmalemmal,
localized Ca^{2+} influx through VGCCs (Ca^{2+} sparklet activity) in αT3–1 gonadotropes (Figure 7, A and B). Acute exposure of αT3–1 cells to GnRH (3nM) increased both Ca^{2+} sparklet activity (nPs) and sparklet site densities (number of Ca^{2+} sparklet sites per μm²) (P < .05, n = 19 cells; Figure 7C). However, pretreatment with dynasore (80μM for 10 min) attenuated both sparklet activity and site densities after the administration of GnRH (3nM) and was not significantly different compared with control or dynasore alone (P > .05, n = 14 cells) (Figure 7C). These results suggest dynamin plays a key role in regulating Ca^{2+} influx through VGCC by engaging the actin cytoskeleton.

**Nicardipine and dynasore are equivalent in attenuating the GnRH-induced whole-cell calcium signal**

GnRH induces a transient increase in intracellular Ca^{2+} by opening VGCCs and releasing intracellular Ca^{2+} from IP_{3}-mediated internal stores. If the effect of dynasore on Ca^{2+} dynamics is confined strictly to the VGCC, then one would predict that its impact on the whole cell Ca^{2+} signal as measured by fura 2 would be equivalent to the VGCC antagonist nicardipine. Consistent with earlier studies (30, 40, 41), the attenuated Ca^{2+} signal in the presence of nicardipine reflects the loss of Ca^{2+} entry via VGCCs and the retention of the IP_{3}-mediated release of intracellular Ca^{2+} stores. Dynasore led to an equivalent reduction in the whole cell Ca^{2+} signal and the combination of nicardipine and dynasore was not additive, suggesting a common site of action. Thus, like nicardipine, the effects of dynasore on GnRH-induced Ca^{2+} dynamics appear to be specific to the VGCC with little or no impact on IP_{3}-mediated Ca^{2+} release. Importantly, these data are entirely consistent with the effect of dynasore to attenuate ERK but not JNK activation in response to GnRH (see Figure 4), because the former is dependent on Ca^{2+} entry via VGCC and the latter dependent on IP_{3} mobilization of intracellular Ca^{2+} (Figure 8) (6).

**Dynamin facilitates GnRH-mediated ERK activation upstream of PKC**

To gain a better mechanistic understanding of dynamin, we next examined where in the signaling cascade dynasore is working to suppress ERK phosphorylation after GnRHR activation. To investigate this, αT3–1 cells were pretreated with 0 or dynasore for 30 minutes and then treated with either 0, GnRHa, or PKC activator PMA for 10 minutes. As expected, ERK phosphorylation was detected 10 minutes after GnRH and PMA treatment. After dynasore treatment, PMA-mediated ERK phosphorylation remained unchanged (Figure 9). However, dynasore treatment significantly decreased GnRH-mediated ERK phosphorylation. These results suggest that dynamin is working upstream of PKC to mediate ERK activation after GnRHR activation.
Figure 7. Inhibition of dynamin decreases GnRH-dependent Ca^{2+} influx via L-type Ca^{2+} channels in αT3–1 cells. A, Representative TIRF images showing localized Ca^{2+} influx in αT3–1 cells treated with GnRH (3nM) with or without dynasore pretreatment (80μM for 10 min). B, Representative fluorescence traces showing time courses of Ca^{2+} influx in αT3–1 cells exposed to GnRH in the presence or absence of dynasore. The scale bar provides reference for changes in [Ca^{2+}]_{i} during the traces (see Materials and Methods). C, Plot of Ca^{2+} sparklet site activities (nP_s).
In the current study, we have identified dynamin as a key signaling intermediate linking GnRHR activation to actin remodeling, VGCC activity and ERK activation. We found that upon GnRHR activation, dynamin associates with cortactin to engage the actin remodeling events. However, pharmacological inhibition of dynamin GTPase activity attenuates GnRH-induced actin polymerization. Consistent with the loss of actin reorganization, dynasore also decreased Ca$^{2+}$ influx through VGCCs and consequently reduced ERK phosphorylation. Thus, dynamin mechano-GTPase activity appears to be important in coupling GnRHR signaling to the actin cytoskeleton and VGCC activity to facilitate ERK activation.

Although dynamin plays a central role in budding endocytic vesicles, it is clear that the PRD of this protein serves as a scaffold for actin interacting proteins. Consistent with this, we demonstrate that GnRHR activation leads to redistribution of dynamin and subsequent association with cortactin at the leading edge of $\alpha$T3–1 cells. Previous work from our group has highlighted the necessity of cortactin in facilitating GnRH-mediated engagement of the actin cytoskeleton in gonadotropes (16). Beyond gonadotropes, other studies have also shown the importance of dynamin and cortactin association in membrane ruffles (18), podosomes (17), and actin comets (19). Interestingly, data show that after association, cortactin appears to stimulate GTPase activity of dynamin, and their association can form a dynamic actin filament remodeling complex (37). Furthermore, there is evidence linking the GTPase activity of dynamin to the formation of F-actin-rich cellular structures (17, 42). In support of this, we show that inhibition of dynamin GTPase activity, using dynasore, blunts GnRH-induced actin remodeling. These data are consistent with overexpression of dominant negative dynamin-K44A that attenuates formation of dendritic spines of neurons (25), dynamic cortical ruffles (26, 27), focal adhesions (43), and podosomes. Although cortactin is a likely candidate for stimulating GTPase activity of dynamin, necessary for actin reorganization, we cannot rule out a direct functional interaction of dynamin and

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found that GnRH-induced actin engagement is required for ERK activation (12), whereas activation of JNK was not affected (Supplemental Figure 1). The importance of GnRH facilitating both actin assembly and ERK activation was also evident in HEK293 cells (53). Interestingly, this preferential suppression of ERK but not JNK activation is seen in a similar manner after the inhibition of dynamin GTPase activity, with similar blunted GnRH-mediated actin reorganization phenotypes. Taken together, there appears to be an interdependence between actin reorganization and ERK activation that is regulated by dynamin GTPase activity.

To better understand the actions of dynamin GTPase activity at the level of MAPK activation, we used an approach that is based on a combination of voltage-clamp electrophysiology and TIRF microscopy that allowed for visualization of Ca2+ influx through VGCCs. Our data demonstrate that dynasore exposure not only decreased the GnRH-mediated increase in Ca2+ sparklet activity, but also the Ca2+ sparklet site density. This potentially explains why dynasore attenuates ERK activation while JNK activation remained unaffected. JNK activation is known to be mediated by IP3 calcium stores, not VGCCs (6). Although it is known that activation of ERK by GnRH requires influx of extracellular Ca2+ through VGCCs (6), we are the first to show a role for dynamin regulating ERK activation through VGCCs.

One question still unanswered is precisely where in the cell signaling cascade dynamin is engaging the actin cytoskeleton and associating with cortactin after GnRHR activation to facilitate Ca2+ influx through VGCCs. Our data suggest that dynamin is working downstream of GnRHR activation but upstream of PKC, because direct stimulation of PKC with PMA leads to ERK activation in the presence of dynasore. Consistent with these findings, our previous work in αT3–1 cells has shown that actin cytoskeletal reorganization is also working downstream of the GnRH but upstream of both PKC and VGCCs (30). These data suggest that ERK activation in gonadotropes is at least partially dependent on the ability of dynamin to associate with cortactin and engage the actin cytoskeleton to then drive PKC-dependent activation of VGCCs. Future studies are necessary to examine how dynamin GTPase activity and the actin cytoskeleton influence the trafficking of PKC in gonadotropes to drive VGCC activity.

In summary, our data support a model by where dynamin is a multifunctional protein not only involved in regulating membrane remodeling through the actin cytoskeleton but also mediating cell signaling events important in gonadotropin subunit synthesis. We propose a model where through GTP hydrolysis, dynamin associates with
Dynamin regulates actin dynamics and ERK activity in gonadotropes by dynamin-dependent VGCC activity and subsequent ERK activation. This mechanism displays a novel role for dynamin that may underlie how gonadotropes maintain adequate sensitivity to incoming neuroendocrine stimulation to produce a physiological response and alignment towards anterior pituitary microvasculature.

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