Cytoskeletal Reorganization Dependence of Signaling by the Gonadotropin-releasing Hormone Receptor*

Received for publication, September 4, 2003, and in revised form, October 13, 2003  
Published, JBC Papers in Press, October 14, 2003, DOI 10.1074/jbc.M309827200

Lindsay Davidson, Adam J. Pawson, Robert P. Millar, and Stuart Maudsley‡

From the Medical Research Council Human Reproductive Sciences Unit, Edinburgh Royal Infirmary, the University of Edinburgh Chancellor’s Building, 49 Little France Crescent, Edinburgh EH16 4SB, Scotland, United Kingdom

Activation of classical G protein-coupled receptors (GPCRs) like the mammalian gonadotropin-releasing hormone receptor (GnRHR) typically stimulates heterotrimeric G protein molecules that subsequently activate downstream effectors. Receptor activation of heterotrimeric G protein pathways primarily controls intermediary cell metabolism by elevation or diminution of soluble cytoplasmic second messenger molecules. We have demonstrated here that stimulation of the GnRHR also results in a dramatic change in both cell adhesion and superstructural morphology. Gonadotropin-releasing hormone (GnRH) receptor activation rapidly increases the capacity of HEK293 cells expressing the GnRHR to remain matrix-adherent in the face of fluid insults. Coinciding with this profound elevation in matrix adherence, we demonstrated a GnRH-induced alteration in both cell morphology and the de novo generation of polymerized actin structures. GnRH induction of cytoskeletal remodeling was correlated with significant increases in the tyrosine phosphorylation status of a series of cytoskeletal associated proteins, e.g. focal adhesion kinase (FAK), c-Src, and microtubule-associated protein kinase (MAPK or ERK1/2). The activation of the distal downstream effector ERK1/2 was demonstrated to be sensitive to the disrupters of cytoskeletal rearrangement, cytochalasin D and latrunculin B. In addition to their classical signaling through heterotrimeric G proteins, many GPCRs initiate activation of the extracellular signal-regulated protein kinase (ERK1/2)-MAP kinase cascade by inducing the tyrosine phosphorylation of proteins that serve as scaffolds for the plasma membrane recruitment of guanine nucleotide exchange factors for monomeric G proteins or for the organization of other signaling molecules. In addition to plasma membrane scaffolding proteins, another site of signaling protein nucleation is the cytoskeletal contact point with the plasma membrane. G protein-coupled receptor stimulation results in a rapid increase in the tyrosine phosphorylation status of docking proteins, such as Shc (1, 2) and Gab1 (3), or of tyrosine kinases, such as the epidermal growth factor or platelet-derived growth factor receptors (3). In many cell types these tyrosine phosphorylation events are sensitive to the inhibition of Src family non-receptor tyrosine kinases (2–4), demonstrating the pluriportant capacity of this molecule. The type I GnRH receptor expressed in the murine Lβ2T2 gonadotrope cell line typically couples to the heterotrimeric Goαq G protein resulting in the activation of PLC-β causing the subsequent activation of protein kinase C (PKC) and intracellular Ca2+ mobilization. It is also apparent that the GnRH receptor, like many other heptahelical G protein-coupled receptors, can also activate monomeric G protein molecules (for review see Ref. 5). The activation of these monomeric pathways has been shown to mediate important effects of GnRH in gonadotropes such as control of gonadotropin hormone subunit transcription (6). The role of tyrosine kinase molecules in GPCR-mediated ERK1/2 activation has been well documented; however, the proximal signaling events whereby these receptors initiate tyrosine phosphorylation remain poorly understood. Several lines of data have demonstrated focal adhesion family kinases and receptor tyrosine kinases, both of which regulate the activity of Src kinases, as proximal mediators of GPCR-induced cellular tyrosine phosphorylation. Focal adhesion tyrosine kinases (FAKs) are non-receptor tyrosine kinases that compose part of the plasma membrane focal adhesion complex. These protein signaling complexes assemble on integrin heterodimers following integrin engagement of extracellular matrix (ECM) proteins. The transmembrane integrin family of cell adhesion molecules mediates cellular contacts to the extracellular matrix. These cell surface receptors are vital for the control of regulating cell motility, polarity, growth, and survival (7). Following recruitment, FAKs auto-phosphorylate and provide docking sites for several signaling proteins, including c-Src and Grb2. Specific signaling pathways, which critically depend on localized integrin activation, have also been reported. Integrin signaling, via actin... 

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† To whom correspondence should be addressed. Tel.: 131-242-6265; Fax: 131-242-6231; E-mail: s.maudsley@hrsu.mrc.ac.uk.

‡ The abbreviations used are: GPCRs, G protein-coupled receptors; GnRHR, gonadotropin-releasing hormone receptor; FAK, focal adhesion kinase; MAPK, microtubule-associated protein kinase; ERK, extracellular signal-regulated kinase; PLC-β, phospholipase C-β; GnRH, gonadotropin-releasing hormone; PKC, protein kinase C; HA, hemagglutinin; DPBS, Dulbecco’s phosphate-buffered saline; BAPTA-AM, 1,2-bis(2-aminoethoxyethane-N,N,N’,N’-tetraacetic acid)-acetoxymethyl; EGFR, epidermal growth factor receptor; GEFs, guanine nucleotide exchange factors; ECM, extracellular matrix.
reorganization, at cell leading edges can activate Cdc42 and Rac1 monomeric G proteins, resulting in membrane extension (8). Matrix-integrin binding has also been shown to profoundly control the activity of Src-like kinases, mPurk, PKC, and GSK-3β (9, 10). In addition to their direct signaling function, integrin-linked actin-binding proteins attach to other signaling molecules and function as signaling nexi in a similar way to the aggregation of proteins around activated receptor tyrosine kinase (11).

In many cell types, stimulation of Goα- or Gqα-coupled receptors causes FAK activation (12–14). This activation is cell adhesion-dependent, because disruption of focal adhesions prevents the response (15). In neuronal cells, stimulation of either lysophosphatidic acid or bradykinin receptors activates the calcium-regulated FAK family kinase, Pyk2 (16), and overexpression of Pyk2 mutants that are either catalytically inactive or unable to bind to c-Src prevents GPCR-induced ERK1/2 activation (4, 16). However, there appears to be great plasticity in downstream GPCR signaling as in other systems GPCRs-mediated ERK1/2 activation is apparently dissociable from FAK phosphorylation (13–14).

Thus utilization of signaling scaffolds can vary between cell types, for example in PC-12 cells GPCR-mediated ERK1/2 activation was almost exclusively focal adhesion-dependent, whereas in rat 1 fibroblasts it was almost exclusively receptor tyrosine kinase-dependent. In HEK293 cells, both scaffolds contributed to the GPCR signal (17). In each case, however, GPCR-stimulated ERK1/2 activation was sensitive to Src kinase inhibitors, suggesting that a critical role of both scaffolds is to support the GPCR-induced activation of Src family nonreceptor tyrosine kinases. In this paper we have identified that GnRH-induced activation of the microtubule-associated protein kinase (MAPK), compared with PKC activation and Ca²⁺ mobilization, is dependent upon a distinct set of circumstances based around differential protein-protein interactions. It appears that an alteration of cellular morphology and reorganization of the cytoskeletal structure trigger the activation of MAPK via a focal adhesion-based scaffolding system, whereas the GnRH control of Ca²⁺/PKC activity was largely independent of this plasma membrane-limited action.

**EXPERIMENTAL PROCEDURES**

**Materials**—The Src kinase-specific inhibitor PP2, the general tyrosine kinase inhibitor herbimycin-A, the EGFR-specific inhibitor tyrphostin AG4178, cytochalasin D, latrunculin B, the PLC-β inhibitor U73122, wortmannin, the PKC inhibitors Ro-31-8200 and GF109203X and the MEK1/2 inhibitor PD98059 were all obtained from Calbiochem. GnRH I, BAPTA-AM, and the synthetic tetrapeptides RGDS and RGES and the Src kinase-specific inhibitor PP2, the general tyrosine kinase inhibitor herbimycin-A, the EGFR-specific inhibitor tyrphostin AG4178, cytochalasin D, latrunculin B, the PLC-β inhibitor U73122, wortmannin, the PKC inhibitors Ro-31-8200 and GF109203X and the MEK1/2 inhibitor PD98059 were all obtained from Calbiochem. GnRH I, BAPTA-AM, and the synthetic tetrapeptides RGDS and RGES were obtained from Sigma. The cDNAs for hemagglutinin (HA)-tagged dominant negative monomeric G protein cDNA clones were obtained from Alan Hall. The Myc-tagged FAK cDNA clones were obtained from Eisuke Nishida.

**Cell Culture and Transfection**—HEK293 cells stably expressing the rat type 1 GnRH receptor (GnRHR) generated within our laboratory (designated SCL60) (56) were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum, 2% glutamine, 1% penicillin (10,000 units/ml)/streptomycin (10,000 mg/ml) at 37 °C in a humidified 5% CO₂ atmosphere. Transient transfections of SCL60 cells were performed by Ca²⁺-phosphate precipitation. Prior to transfection appropriately transfected or untransfected cells were incubated in serum-free media (Dulbecco’s modified Eagle’s medium, 2% glutamine, 1% penicillin/streptomycin, 10 μM HEPES) for 16 h. Agonist stimulations were performed at 37 °C in serum-free media following preincubation with chemical inhibitors as described in the figure legends.

To actively displace integrins from their cell matrix attachments, SCL60 cells were incubated in serum-free media with the RGDS or RGES tetrapeptides at a 1 μM final concentration for 16 h. At the time of agonist stimulation, any displaced and detached cells were collected with any adherent cells by centrifugation at 1000 × g for 5 min.

**Immunoprecipitation and Immunoblotting**—Following ligand stimulation, confluent monolayers were placed on ice, washed once with ice-cold Dulbecco’s phosphate-buffered saline (DPBS), and lysed in a Nonidet P-40-based solubilization buffer (250 mM NaCl, 50 mM HEPES, 0.5% Nonidet P-40, 10% glycerol, 2 mM EDTA, pH 8.0, supplemented with 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin). Solubilized lysates were clarified by centrifugation at 10,000 × g for 15 min and diluted to an approximate concentration of 1 mg/ml of total protein. Where appropriate, a 40-μl aliquot of clarified whole-cell lysate was mixed with an equal volume of 2 × Laemmli sample buffer and resolved by SDS-PAGE for confirmation of plasmid expression or determination of protein expression or phosphorylation by specific protein immunoblots. Immunoprecipitation of endogenous active or inactive extracellular signal-regulated kinase (ERK) from clarified whole-cell lysates was performed using 20 μl of a 50% anti-pyruvate kinase antibody (Santa Cruz Biotechnology) incubated with the clarified whole-cell lysate in addition to 20 μl of a 30% slurry of protein G plus/protein A-agarose (Calbiochem) for 16 h at 4 °C with continuous agitation. Immunoprecipitation of the transiently transfected HA-tagged FAK constructs was achieved using 20 μl of a 50% anti-HA-agarose preconjugate slurry (Santa Cruz Biotechnology) added to the clarified whole-cell lysate with constant agitation at 4 °C for 16 h. Immunoprecipitation of Myc-tagged proteins was achieved by incubating clarified whole-cell lysates with 20 μl of a 50% anti-Myc preconjugate slurry (Santa Cruz Biotechnology) with constant agitation at 16 h at 4 °C. Immune complexes were collected by centrifugation at 20,000 × g for 10 min and washed twice in ice-cold Nonidet P-40-based solubilization buffer described previously. After washing, 20 μl of 2 × Laemmli sample buffer was added to each collected immune complex. Immunoprecipitates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane (PerkinElmer Life Sciences) for protein immunoblotting. Polyvinylidene difluoride membranes were blocked in a 4% bovine serum albumin, 50 mM Tris-HCl, pH 7.0, 0.05% Tween 20, and 0.05% Nonidet P-40 blocking solution. Immunoblotting of endogenous active or inactive extracellular signal-regulated kinase (ERK) from clarified whole-cell lysates was performed using a 1:1000 dilution of rabbit anti-human ERK (up phosphorylated, New England Biolabs) or 1:1000 dilution of rabbit anti-human active ERK (phosphorylated, New England Biolabs) antisera. Visualization of the active or inactive ERK was achieved by addition of a 1:15,000 dilution of alkaline phosphatase-conjugated polyclonal anti-rabbit IgG as a secondary antibody (Sigma). Each alkaline phosphatase-labeled protein was visualized using an enzyme-linked chemifluorescence reaction (Amersham Biosciences) and quantified using a Storm 860 PhosphoImager. Expression of endogenous FAK or FAK protein present in an immunoprecipitation was visualized using a rabbit anti-human FAK polyclonal IgG (Santa Cruz Biotechnology) at a 1:1000 dilution with a 1:15,000 dilution of alkaline phosphatase-conjugated polyclonal anti-rabbit IgG as a secondary antibody (Sigma). Detection of HA-tagged FAK either from whole-cell lysate extracts or from immunoprecipitations was achieved using a mouse anti-hemagglutinin 12CA5 monoclonal IgG1 (Roche Applied Science) at 1:1000 with a 1:15,000 dilution of alkaline phosphatase-conjugated polyclonal anti-mouse IgG as a secondary antibody (Sigma). Detection of the tyrosine phosphorylation status of proteins from whole-cell lysates or immunoprecipitations was achieved using a mouse anti-phosphotyrosine PY99 monoclonal (Santa Cruz Biotechnology) at a 1:1000 dilution with a 1:15,000 dilution of alkaline phosphatase-conjugated polyclonal anti-mouse IgG as a secondary antibody (Sigma). Immunoblotting of un-phosphorylated c-Src protein was performed using a rabbit anti-human Src polyclonal (Santa Cruz Biotechnology) at a 1:1000 dilution with a 1:15,000 dilution of alkaline phosphatase-conjugated polyclonal anti-rabbit IgG as a secondary antibody (Sigma). The specific tyrosine phosphorylation status of c-Src was investigated using anti-human polyclonal antisera raised against the specific phosphorylated forms Tyr-418 (autophosphorylation site) for Src from N source International). These were both employed at a 1:1000 dilution with a 1:15,000 dilution of alkaline phosphatase-conjugated polyclonal anti-rabbit IgG as a secondary antibody (Sigma).

**Immunocytochemistry and Confocal Laser Microscopy**—Confocal laser microscopy was performed on a Zeiss LSM510 laser-scanning microscope using a 40 × 1.4 numerical aperture oil immersion lens (Zeiss). SCL60 cells were plated upon poly-L-lysine-treated 8-well chamber slides (Nunc-Nalgene) at a density of 60,000 cells per chamber and serum-starved (16 h) before cellular stimulation. After stimulation cell monolayers were washed twice with ice-cold DPBS (with Ca²⁺/Mg²⁺) and
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RESULTS

GnRH Stimulation of the Type I GnRH Receptor Enhances the Adhesive Capacity of Fibroblast Cells—The cellular effects of G protein-coupled receptor activation are typically thought to be solely associated with effects upon intermediary cell metabolism primarily through the generation of cytoplasmic soluble second messenger molecules. However, we have noted that upon stimulation of the GnRHR there are rapid changes in the morphology and adherence of the cells, suggesting perturbation of cytoskeletal structural organization. After stimulation of HEK293 cells stably expressing the rat type I GnRHR, there was a rapid increase in cellular adherence (Fig. 1, panel a), and this effect was dose-dependent with GnRH I (data not shown). The adherence effect was measured by assessing the capacity of a mechanical agitation insult to allow detachment of cells from the growth plate surface. The cellular adherence effect was also induced by other GnRH agonist molecules such as GnRH II but not by GnRH antagonist molecules such as antagonist 135-18 (data not shown and see Ref. 19). Peirucination of the SCL60 cells with a classical antagonist, i.e. antagonist 135-18 (30 min, 1 μM), abrogated the ability of GnRH I to induce cellular adherence. Stimulation of HEK293 cells not expressing any endogenous GnRH receptor (wild-type HEK293) resulted in no significant effect upon the capacity of the cells to be detached by the DPBS insult.

In exploratory studies on this phenomenon, we noted that when used at a high nonspecific dose (5 μM) the tyrosine kinase inhibitor herbimycin-A was able to greatly attenuate the capacity of GnRH stimulation to increase the adherence of the receptor-expressing SCL60 cells. When the SCL60 cells were pretreated with the selective Src kinase inhibitor PP2 (1 μM, 30 min) there was a significant attenuation of the GnRH-induced cell adherence phenomenon similar to the effect of herbimycin-A (Fig. 1, panel c). Inhibitors of both the epidermal growth factor receptor tyrosine kinase activity (tyrphostin AG1478, 100 nM) and Janus kinase activity (tyrphostin AG490, 100 nM) failed to inhibit significantly the increased cell adherence induced by GnRH (data not shown). Thus it appears that the tyrosine kinase activity of c-Src was involved in the ability of GnRH to alter dramatically the interaction of the cell with the external environment. The non-receptor tyrosine kinase c-Src has been demonstrated to be physically associated with cell structural components such as cytoskeletal stress fibers and focal adhesion complexes (for review see Ref. 20); therefore, we investigated whether the GnRH-induced cell adherence was sensitive to chemical disrupters of the dynamic nature of the cell superstructure. Incubation of the SCL60 cells in 1 μM cytochalasin-D for 60 min before GnRH I stimulation inhibited the ability of GnRH I to increase cellular adherence (Fig. 1, panel d). Therefore these data suggest that both the induction of cellular tyrosine kinase activity and the integrity of the cell superstructure are required for the capacity of GnRHR stimulation to induce an alteration of the interaction of the cell with its local environment.

GnRH-induced Alterations of HEK293 Cell Superstructure—We observed that within 10–30 min of GnRH stimulation, SCL60 cells became considerably more squamous, showing signs of increased spreading and lamellodia formation, resulting in a less stellate morphology than cells in unstimulated phase contrast fields (Fig. 2, panels 1 and 2). These morphological changes were accompanied by the redistribution of actin to the remodeled plasma membrane and to a lesser degree into fibrous structures (Fig. 2, panels 3 and 4). These redistribution of actin was accompanied by the redistribution of microtubules (Fig. 2, panels 7 and 8). The full generation of these structural features occurred within 30 min of cellular stimulation. Therefore, GnRH induction of the cellular adherence effect is accompanied by extensive reassembly of the cytoskeleton. The generation of the ultrastructural changes in the cytoskeletal structure of the SCL60 cells appeared to be reliant upon the catalytic activity of c-Src. When cells were pretreated with 5 μM PP2, there was shown to be no specific GnRH-induced alteration in the general cell morphology both under phase contrast (Fig. 2, panel 9, unstimulated; panel 10, stimulated) and at the level of actin reorganization (Fig. 2, panel 11, unstimulated; panel 12, stimulated).

GnRH-induced Activation of Cellular Structure Associated Proteins—Recent reports have identified that many cellular structural proteins also can play an important role in the intracellular signaling of GPCRs. We therefore investigated the well documented ability of the GnRH receptor to activate the microtubule-associated protein kinase (also known as the mitogen-activated protein kinase, MAPK). Upon GnRH stimulation of the SCL60 cells there is a potent dose- (not shown) and time-dependent activation of the MAPK ERK1/2 isoforms (Fig. 3, panel a). The possible involvement of two other structurally regulated proteins that possess tyrosine kinase activity, the focal adhesion tyrosine kinase (FAK), and the non-receptor tyrosine kinase c-Src were investigated. The involvement of these two signaling intermediates on the effects upon cell superstructure of GnRH (Figs. 1 and 2) was suggested by the potent effects of c-Src tyrosine kinase inhibitors upon the ad-
herence phenomenon and also by the profound alteration of the cytoskeletal proteins actin and tubulin. Immunoprecipitation of cellular FAK from cells after GnRH stimulation revealed a potent increase in the degree of its tyrosine phosphorylation status (Fig. 3), indicative of the activation of its intrinsic tyrosine kinase activity. Upon engagement of the FAK protein with the \(/ H9252^{/}H11003/INT\) integrin molecule at the focal adhesion complex, the tyrosine kinase activity of FAK is activated and thus begins to autophosphorylate itself upon key tyrosine residues involved in signal protein complex assembly. Thus GnRH stimulation of the SCL60 cells resulted in a significant time-dependent increase in the intrinsic tyrosine kinase activity of FAK (Fig. 3, panel b).

When studying the effect of GnRH upon the non-receptor tyrosine kinase Src, we employed two antisera that recognize two specific phosphorylated forms of the tyrosine kinase, i.e., anti-Tyr\(^{418}\) c-Src antisera recognize the specifically autophosphorylated form of Src. Tyrosine phosphorylation of the Tyr\(^{418}\) residue occurs by the actual tyrosine kinase of Src itself and serves to stabilize the active conformation of the c-Src kinase domain. The anti-Tyr\(^{529}\) polyclonal sera was used to investigate the phosphorylation status of the regulatory tyrosine in the carboxyl terminus of Src. In the inactive Src molecule the Tyr\(^{529}\) is normally phosphorylated and engaged with the Src homology 2 domain of the amino terminus of Src. The dephosphorylation of this residue is a typical prerequisite of the cellular activation of c-Src. In a similar time course to the activation of FAK, GnRH stimulation resulted in the elevation of the degree of Tyr\(^{418}\) c-Src tyrosine phosphorylation (Fig. 3, panel c). GnRH stimulation also induced an initial reduction of the tyrosine phosphorylation status of the Tyr\(^{529}\) residue (Fig. 3, panel d). Thus it appears that with GnRH receptor activation there is a concerted alteration of the activity status of many intracellular proteins concerned with and physically linked to the cell superstructure. The coordinated activation of these proteins may play a significant role in the adherent capacity of the SCL60 cells.
proteins therefore may represent a coherent form of GnRH receptor signaling connected to the maintenance of the cytoskeletal architecture of the cells expressing the type I GnRHR. Therefore, we investigated whether the temporally coordinated activation of the cytoskeletal-associated proteins forms a signaling network that could be entrained by GnRH-mediated alterations in the cell superstructure.

GnRH-mediated Activation of MAPK, FAK, and c-Src Occurs via a Similar Molecular Mechanism—GnRH activation of ERK1/2-MAPK was relatively insensitive to inhibition of PLC-β or phosphatidylinositol 3-kinase activity (Fig. 4, panel a). In addition, chelation of intracellular Ca²⁺ with BAPTA-AM had no significant effect upon GnRH-mediated ERK1/2 activation. Activation of protein kinase C also appeared to be unrelated to the elevation of ERK1/2 activity in response to GnRH.

In contrast, preincubation of SCL60 monolayers with the Src kinase inhibitor PP2 significantly attenuated the ERK1/2 activation (Fig. 4, panel a). Unlike other reported ERK1/2 activation mechanisms, we failed to observe any great involvement of the tyrosine kinase activity of the epidermal growth factor receptor as evidenced by the inability of tyrphostin AG1478 to attenuate the ERK1/2 activation response. When the same panel of chemical inhibitors was tested against the GnRH-induced elevation in the activity of FAK and c-Src, a similar pattern was observed (Fig. 4, panels b and c) except for the inhibitory activity of the MEK1/2 inhibitor PD98059 that did not significantly affect the ability of the GnRH to induce tyrosine kinase activation at either FAK or c-Src. Thus it appears that the activation of the cytoskeleton-linked proteins may occur in a correlated manner, suggesting a physical and consequential link that underlies the control of cellular structure in its relation to the change of cell surface-matrix contact. Therefore, we investigated whether the synergistic activation of these proteins was dependent upon the physical integrity of the focal adhesion and cytoskeletal matrix.

GnRH-mediated Activations of MAPK, FAK, and c-Src Are Dependent Upon Intact Cytoskeletal Structure—We employed several chemically distinct agents to disrupt both the cytoskeletal cell matrix and also specifically the integrin-based focal adhesion complexes. We first studied the effects of cytochalasin D (a de-polymerizer of actin structures) and latrunculin B (prevents de novo actin polymerization) upon GnRH-mediated ERK1/2, FAK, and c-Src activation. As demonstrated in Fig. 5 (panel a), preincubation of the SCL60 cells with 1 μM cytochalasin D or 1 μM latrunculin B significantly attenuated the

GnRH I stimulation (100 nM) and eventual fixing. Panel 1 is a representative phase contrast image of unstimulated SCL60 cells and panel 2 depicts representative GnRH I-stimulated cells that display a considerably more squamous appearance with fewer stellate projections and much greater total surface area. Panels 3 and 4 depict permeabilized SCL60 cells stained for β-actin (FITC-green) in the absence of GnRH (panel 3) and after 30 min of GnRH I stimulation (panel 4). The profound generation of actin-rich plasma membrane extensions (lamellopodia) and, to a lesser extent, fibers is evident in the GnRH I-treated cells compared with those not stimulated in which the actin is not as significantly polymerized. A similar effect of GnRH upon actin mobilization within the cell was evident also when actin was visualized using a different methodology, i.e. Alexafluor-568-phalloidin (red; panel 5, unstimulated; panel 6, stimulated). Panels 7 and 8 depict permeabilized SCL60 cells stained for tyrosine tubulin (FITC-green) in the absence (panel 7) and after stimulation with GnRH I (panel 8). Without GnRH stimulation there is a diffuse pattern of tyrosine tubulin staining, yet after GnRH receptor activation the generation of multiple microtubule fibers is clearly evident. Pretreatment of SCL60 cells with 5 μM PP2 (30 min, panels 9–12), before addition of GnRH I (100 nM), and immunostaining for β-actin (FITC-green) significantly inhibited the agonist-induced changes in cell morphology (panel 9, unstimulated; panel 10, GnRH stimulation) and the actin cytoskeleton (panel 11, unstimulated; panel 12, stimulated).
capacity of GnRH to elevate the amount of active ERK1/2. When we assessed the GnRH-induced increase in FAK auto-
phosphorylation, we noted that the two actin-disrupting agents
were extremely potent at inhibiting the actions of GnRH I (Fig.
panel b). In a similar manner to their effects upon GnRH-
induced FAK phosphorylation, both cytochalasin D and latrun-
culin B inhibited the GnRH-mediated autophosphorylation of
c-Src at tyrosine 418 (Fig. 5, panel c). Therefore, it appears that
the coordinated GnRH-induced control of the cytoskeleton-
linked proteins is critically dependent upon the normal func-
tioning of the cellular actin matrix. We next investigated the
role of the assembled focal adhesion complexes, often tethered
to the actin cytoskeleton at the plasma membrane, in the signal
transfer from the GnRH receptor in the SCL60 cells.

Transmembrane integrin receptors typically interact with
the minimum consensus peptide sequences, e.g. Arg-Gly-Asp-
Ser, within the extracellular matrix protein fibronectin. There-
fore, if an excess of an Arg-Gly-Asp-Ser (RGDS) tetrapeptide is
incubated with the cells adherent to an extracellular matrix, it
will effectively displace the extracellular region of the integrin
from the matrix protein resulting in the disruption of the stable
integrin α-β dimer. The disruption of this integrin receptor
dimer typically results in the destruction of the focal adhesion
protein complex. We employed two synthetic tetrapeptides to
affect selectively the integrin-stabilized focal adhesion complexes within the SCL60 cells, i.e. RGDS and the chemically similar RGES as a negative control tetrapeptide. Integrin receptors fail to bind the RGES sequence despite its superficial similarity to the consensus fibronectin attachment sequence. Preincubating the SCL60 cells with either 1 mM RGDS or 1 mM RGES for 16 h in serum-deprived conditions resulted in dramatic differences upon the general state of the cells. SCL60 cells incubated with the RGDS tetrapeptide tended to become easily dislodged from the growth plate, whereas cells treated with RGES remained unaffected. Upon collection of either RGDS- or RGES-treated cells it was noted that, after a 10-min GnRH 1 stimulation, the ability of GnRH to activate ERK1/2 was almost completely inhibited by incubation with the RGDS tetrapeptide and was largely unaffected by the RGES tetrapeptide (Fig. 5, panel d). An almost identical pattern of tetrapeptide activity upon GnRH-mediated FAK or c-Src activation was additionally noted (Fig. 5, panels e and f, respectively). Thus, as with the actin-disrupting agents, the selective disturbance of focal adhesion complexes resulted in the attenuation of the ability of GnRHS to coordinate the activation of the cytoskeleton-linked proteins.

**FAK Acts as a Signaling Nexus for GnRH-induced Cellular Actions**—We have thus shown that a primary function of GnRH is to mobilize the cell superstructure to control signaling functions. The ability of GnRH stimulation to activate FAK suggests the capacity to assemble molecules at these scaffolding molecules tethered to the cell membrane by cytoskeletal structures. Thus we investigated whether GnRH activation results in the formation of protein-protein interactions between the

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**Fig. 4. Chemical sensitivity of GnRH-induced ERK1/2, FAK, and c-Src activation in SCL60 cells.** SCL60 cells after 16 h of serum deprivation were preincubated with various chemical inhibitors of common intracellular signaling pathways before application of 10 nM GnRH I for 10 min (−, no GnRH; +, GnRH stimulation). Cell lysate extracts were then assayed by immunoblot for ERK1/2 activation. Anti-phosphotyrosine immunoprecipitates were assayed by selective immunoblot for both FAK or c-Src activation. ERK1/2 activation (panel a) but neither FAK (panel b) or Src (panel c) activation was inhibited by preincubation with the MEK1/2 inhibitor PD98059 (20 μM for 60 min). ERK1/2 (panel a), FAK (panel b), and c-Src (panel c) activation were unaffected by U73122 inhibition of PLC-γ (20 μM for 60 min), wortmannin inhibition of phosphatidylinositol 3-kinase (100 nM for 30 min), BAPTA-AM chelation of intracellular calcium release (50 μM for 30 min), Ro-31 8220 (100 nM for 30 min), or GF109203X (1 μM for 30 min) inhibition of PKC or tyrphostin AG1478 inhibition of EGFR activity (100 nM for 30 min). ERK1/2 (panel a), FAK (panel b), or c-Src (panel c) activation were all attenuated by preincubation with the c-Src kinase inhibitor PP2 (5 μM for 30 min).
cytoskeletal associated proteins that are coordinately stimulated by GnRH in SCL60 cells. In Fig. 6 it is evident that GnRH induces an active, time-dependent association between c-Src and FAK (panel a). When the activation status of the FAK-associated c-Src was studied, it was noted that the temporal change in activation status of the FAK-associated c-Src followed a similar pattern to that of the total c-Src association with FAK (panel b). This would suggest that GnRH induces a largely de novo association and subsequent activation of c-Src with FAK. In contrast, in the absence of GnRH ligand there was a considerable level of inactive ERK2 constitutively associated with FAK (Fig. 6, panel c). When the Tyr mutant FAK isoforms (Y397F and Y925F) are co-expressed with Myc-ERK, there is a diminution of the GnRH-induced ERK activation. The Tyr-397 mutant, which displays an attenuated capacity to bind c-Src (21), inhibited the GnRH-induced ERK activation to a greater extent than the Y925F mutant (attenuated in its capacity to bind signaling molecules such as Grb2). This suggests that the capacity of FAK to functionally interact with c-Src is of primary impor-

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![Diagram](https://via.placeholder.com/150)

**Fig. 5.** GnRH-induced activation of ERK1/2, FAK, and c-Src is dependent upon an intact cytoskeletal structure. Serum-deprived SCL60 cells were treated with either 1 μM cytochalasin D (cyto-D) or 1 μM latrunculin B (lat-B) for 60 min before GnRH I stimulation (+, GnRH stimulation: 100 nM, 10 min) and eventual measurement of ERK1/2 activation, FAK tyrosine phosphorylation, or c-Src Tyr-418 phosphorylation. Panel a demonstrates that the ERK1/2 activation by GnRH is blunted by both cytochalasin D and latrunculin B. Both these cytoskeletal disrupting agents also significantly attenuate the GnRH-induced FAK and c-Src activation (panels b and c, respectively). Preincubation of serum-deprived SCL60 cells with 1 mM RGDS but not 1 mM RGES tetrapeptide for 16 h before GnRH I stimulation (100 nM, 10 min) resulted in a significant inhibition of the ability of GnRH to activate ERK1/2 (panel d), FAK (panel e), and c-Src (panel f). Each bar represents mean ± S.E. data gathered from at least three individual experiments.

To further reinforce this proposal, we employed point-mutated forms of FAK in which several tyrosines, forming docking sites for Src homology 2-domain (SH2 domain)-containing proteins, were mutated to phenylalanine. These mutations prevent potential tyrosine phosphorylation and abrogate subsequent association of SH2 domain-containing proteins with FAK. In addition we employed a kinase-deficient mutated form of FAK to demonstrate the requirement of the intrinsic FAK tyrosine kinase domain for the GnRH-mediated cytoskeletal signaling. Transient expression of wild-type HA-tagged FAK with the Myc-tagged ERK construct demonstrates that upon GnRH stimulation and immunoprecipitation of myc-ERK there is a profound elevation of ERK activity (Fig. 7, panel a). However, when the Tyr mutant FAK isoforms (Y397F and Y925F) are co-expressed with myc-ERK, there is a diminution of the GnRH-mediated ERK activation signaling. This suggests that the capacity of FAK to functionally interact with c-Src is of primary impor-
tance with respect to the ability of GnRH to activate ERK in the SCL60 cells, and to a lesser extent with downstream signaling molecules such as Grb2. Overexpression of a FAK mutant in which the ATP-binding arginine (Arg-454) is mutated, and thus cannot act as a tyrosine kinase, also resulted in a blunting of the GnRH-mediated ERK activation (Fig. 7), demonstrating that the capacity for GnRH to activate the intrinsic tyrosine kinase activity of FAK is required for the efficient activation of ERK.

Thus we have shown that the rearrangement of the cell superstructure is important for eventual downstream signaling to proteins such as c-Src and ERK. This form of signaling is channeled through the assembly of signaling complexes at the FAK present at plasma membrane focal adhesions. However, the mechanism by which GnRH first initiates the activation of FAK remains unresolved. Clearly there is extensive re-modeling of the plasma membrane of the cells as shown in Fig. 2 (panels 2, 4, and 6), and if this allows the generation of enhanced cell-matrix contact seen in Fig. 1, then it is possible that the generation of this remodeling event may underlie the initial activation of FAK. Indeed, it has been recognized that the generation of additional focal adhesions and the generation of polymerized actin structures result in elevated FAK activity via increases in the number of integrin-FAK interactions. A primary mechanism by which transmembrane receptors can regulate cell membrane morphology is via the activation of the Rho family of monomeric G proteins (for review see Ref. 22). We therefore investigated whether GnRH-mediated FAK activation was dependent upon Rho family G protein activation. By using the HA-tagged FAK, we co-expressed dominant negative Rho family G protein mutants (N19RhoA and N17Rac1), then immunoprecipitated the expressed FAK, and studied the small G protein mutants effects upon the GnRH-induced FAK auto-tyrosine phosphorylation (Fig. 8). When the dominant negative RhoA construct is expressed, there is little effect upon the GnRH-induced FAK activation. In contrast when N17Rac1 was expressed, there was a potent inhibition of the GnRH-induced FAK activation suggesting that indeed the plasma membrane remodeling induced by the activation of these monomeric G proteins may indeed be responsible for the GnRH-mediated FAK activation.

GnRH-mediated Signaling Is Divergent between Heterotrimeric and Monomeric G Protein-mediated Signaling—It ap-
pears that the GnRH receptor-mediated activation of the cytoskeletal proteins FAK, Src, and ERK is dependent upon a profound plasma membrane remodeling event. This effect is relatively rapid in onset and is largely insensitive to inhibition of PLC-β activation, yet the activation of PLC-β is considered to be the primary mode of action of the Gαq-coupled GnRH receptor. The Gαq-mediated activation of PLC-β results in subsequent hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol, which in turn elevate intracellular calcium concentrations and activate PKC, respectively. This can be adequately demonstrated by measurement of total free inositol production after GnRH stimulation. In Fig. 9, panels a and b, it can be seen that GnRH induces both a dose- and time-dependent increase in the pool of free inositol phosphates in SCL60 cells. However, as demonstrated previously, there appears to be a divergence in the signaling cascades that mediate PKC activation/Ca^{2+} mobilization and those that control ERK activation (Fig. 4, panel a). Conversely, we also find that cytochalasin D and latrunculin B, both efficient inhibitors of ERK/FAK/Src activation, were completely unable to attenuate GnRH-induced and Gαq-mediated inositol phosphate accumulation (Fig. 9, panel c). In the same experimental paradigm, inhibition of PLC-β with U73122 was able to abrogate almost completely the GnRH-induced inositol phosphate accumulation. This demonstrates that there are two distinct channels of receptor activity entrained upon GnRH interaction with the receptor at the plasma membrane as follows: one in which stimulation of the heterotrimeric Gαq results in alteration of Ca^{2+}/PKC-dependent effects which does not involve plasma membrane remodeling, and one which does involve significant plasma membrane remodeling leading to the formation of signaling complexes required for the activation of mitogen-related pathways (Fig. 10).

**DISCUSSION**

The mammalian type I GnRH receptor is unique among rhodopsin-like GPCRs as it possesses no intracellular cytoplasmic carboxyl-terminal tail. Despite this it can readily couple to multiple heterotrimeric G proteins (23) as well as small monomeric G proteins (6), demonstrating that in the case of this receptor G protein coupling occurs through the three intracellular cytoplasmic loops. The primary mode of GnRH signaling in the gonadotrope occurs through the Gαq heterotrimeric G protein that stimulates the catalytic activity of PLC-β, generating the soluble second messengers diacylglycerol and inositol trisphosphate. These two intermediates then control the activation of protein kinase C isofoms and the liberation of free intracellular calcium, respectively. The activation of the small monomeric Ras-type G proteins typically occurs through a growth factor receptor-induced association at the plasma membrane with guanine nucleotide exchange factors (GEFs) such as Son of Sevenless (Sos) (24). The activation of the Rho/Rac/Cdc42 monomeric G proteins are regulated by essentially the same mechanism as Ras proteins by a series of interactions with both GEFs and with GTPase-activating proteins. However, they are further regulated by another class of regulators, the GDP dissociation inhibitor type of proteins (25, 26). The cytosolic Rho family proteins are complexed with their respective GDP dissociation inhibitor and thus maintained in an inactive GDP-bound form. Upon “activation,” the Rho protein is released from the GDP dissociation inhibitor and is rapidly converted to the GTP-bound form by the action of a GEF.
Termination of the Rho signaling event is mediated by GTPase-activating protein-mediated return to the GDP-bound state of the Rho family protein.

Here we have demonstrated that the type I GnRH receptor when stably expressed in a HEK293 cell background still acts in a similar manner to the pituitary gonadotrope in activating PLC-β. In addition to this classical GnRH-induced cellular activation, interesting rapid effects upon cell adhesion and structural morphology were observed. These effects were both dose- and time-dependent and were also abolished by prior exposure to classical type I GnRH receptor antagonists. Stimulation of control wild-type HEK293 cells not expressing the type I GnRH receptor failed to induce any increase in cell adhesion or alterations in morphology. These findings demonstrate that these actions of GnRH were directly mediated through the GnRH receptor and were not due to any nonspecific effects of the agonists upon the cells. The enhanced adherence of the cells (Fig. 1) could therefore be correlated to the alteration of cytoskeletal actin and also of tyrosine tubulin (Fig. 2). These two effects were both sensitive to disruption of actin polymerization and general tyrosine kinase inhibition. Actin polymerization is typically required for many types of cell motility, e.g. chemotaxis, nerve growth cone movement, cell spreading, and platelet activation (27). The clear GnRH-induced morphological changes seen in Fig. 2 suggest that the membrane has been extruded, into lamellipodia-like structures, to increase surface area contact with the plate surface (Fig. 2, panels 2, 4, and 6). Cell stimulation can activate actin polymerization in several ways, e.g. de novo nucleation of filaments from monomeric actin, severing existing filaments to create uncapped barbed ends, or uncapping existing barbed ends (28). In the present study we have demonstrated that both cytochalasin D (actin de-polymerizer) and latrunculin B (inhibitor of actin polymerization) disrupted the GnRHR-mediated morphological changes suggesting that both the de novo polymerization and de-polymerization/re-organization events are occurring. The activation of c-Src was found to be critical for these morphological changes to occur. It has been shown that Src activation is important for many cellular actions of GnRH (6, 29–30). The morphological changes observed upon GnRH receptor activation were significantly inhibited upon incubation with the Src kinase inhibitor PP2. The non-receptor tyrosine kinase c-Src has long been known to be associated with cytoskeletal element control (31). Originally it was shown that v-Src, the oncogenic form of the non-receptor tyrosine kinase, was located in focal adhesion complexes (32, 33). Focal adhesion complexes are large protein signaling complexes clustered around transmembrane integrin receptors. The ability of integrin receptors to interact with extracellular matrix (ECM) proteins such as fibronectin, vitronectin, or collagen promotes and stabilizes cell adherence and allows for motility. The integrin receptors themselves also connect the ECM proteins to many cytoskeletal elements (34). This was first seen in tumor cells in which introduction of the ECM protein induced the cells to flatten out and resulted in a dramatic reorganization of the cytoskeleton (35, 36). As well as anchoring cells to their substratum, integrins are also important nexi of signaling function. Their effects on cell signaling pathways are mediated by their capacity to control the architecture of the actin cytoskeleton. Early work demonstrated that integrin-mediated ECM engagement could induce specific gene expression and modulation (37, 38), which is typically the preserve of growth factor receptors that possess their own intrinsic tyrosine kinase activity. Both α and β integrin receptors, once engaged to the ECM, dimerize to form αβ heterodimers. This dimerization causes the nucleation of the multiprotein focal adhesion complex containing both cytoskeletal elements (actin, talin, and vinculin) and catalytic signaling proteins (Src, EGFR, and platelet-derived growth factor receptor) and other structural proteins and adapters (Grb2, paxillin, and p130Cas); for review see Ref. 39). Part of this signaling complex is the p125 focal adhesion kinase. FAK undergoes autotyrosine phosphorylation after binding the β integrin, thus producing docking sites for many signaling proteins and adapters such as Grb2 (11), c-Src.
The stimulation of the tyrosine phosphorylation of FAK can induce the assembly of signaling complexes capable of activating signaling pathways required for the control of gene expression through the MAPK pathway (17). In the present study the generation of changes in cell morphology were temporally coincident with a potent activation of the tyrosine autophosphorylation of FAK, elevation of MAPK activity, and a significant elevation of c-Src catalytic activity (Fig. 3). Our model proposes that the formation of ERK-activating signaling complexes could be dependent on cytoskeletal structures. The disruption of the integrin-based focal adhesion assembly of signaling proteins by the functionally distinct cytochalasin D and latrunculin B inhibited GnRH stimulation of the activity status of the cytoskeletal linked proteins (Fig. 4, panels a−c). In addition by disrupting the point of engagement with the extracellular matrix with the ECM antagonist peptide RGDS, the GnRH receptor control over the focal complex signaling assembly was lost (Fig. 4, panels d−f). The eventual downstream stimulation of the MAPK appeared to be directly controlled, in part, by c-Src, as PKC inhibition, intracellular Ca^{2+} chelation, and inhibition of the EGFR tyrosine kinase all failed to inhibit the GnRH-induced ERK activation in SCL60 cells (Fig. 5). Indeed there has been shown to be a multitude of potential mechanisms through which MAPK activation can occur via GPCR stimulation (41). Thus instead, in this paradigm the most important mechanism appears to rely upon the catalytic activity of c-Src, above Ca^{2+} mobilization and PKC activation typically induced by GnRH receptor activation. Previously it has been shown (42−44) that FAK can act as a scaffold for the coordinated assembly of MAPK-signaling entities. We have also shown that under GnRH control both ERK itself and c-Src can associate with the catalytically activated FAK (Fig. 7). Interestingly, along with the increase in cell adherence, altered morphology, and increased FAK phosphorylation, there is a physical elevation of Src interaction with FAK, yet in contrast a subset of ERK protein appears tonically scaffolded to FAK. Thus we have shown that GnRH induces an elevation of Src interaction and subsequent catalytic activation with FAK. In contrast ERK co-associates with FAK in the absence of GnRH stimulation, but like Src, this ERK becomes activated upon GnRH stimulation of the cells. Therefore, we can illuminate in part the dynamics of signaling complex as-

![Image](https://via.placeholder.com/150)

**Fig. 9.** GnRH-induced activation of inositol phosphate turnover is independent of plasma membrane turnover and cytoskeletal reassembly. Panels a−c depict data gathered from SCL60 cells preincubated with myo-[3H]inositol for 48 h in specific inositol-free growth media. Total inositols liberated from the plasma membrane of the cells, released by PLC-β-mediated cleavage, were collected by chromatographic techniques previously described under “Experimental Procedures.” Panels a and b demonstrate that in SCL60 cells GnRH I induces a potent and protracted elevation of free [3H]inositol phosphates. Panel c demonstrates that the GnRH I-induced elevation of free inositol phosphates is sensitive to inhibition of PLC-β with U73122 but is almost completely insensitive to disruption of cytoskeletal rearrangement as neither cytochalasin D nor latrunculin B pretreatment has any effect upon GnRH-induced inositol phosphate production (+, no stimulation; +, GnRH stimulation: 100 nM, 10 min). Each bar represents the mean ± S.E. of GnRH-induced total inositol phosphate data gathered (fold over basal) from at least three individual experiments.
semblably and resultant MAPK activation in HEK293 cells at the sites of focal adhesions. Over recent years it has been demonstrated that the canonical and well conserved MAPK activation cascade is often organized in a modular fashion. Large scaffolding proteins, such as Ste5 (45), β-arrestin (46), and JIP (47), can hold several members of the canonical MAPK cascade. These three distinct proteins can all scaffold and preorganize MAPK kinase kinases (Ste11, Raf, and Ask-1), MAPK kinases (Ste7, MEK1/2, and MKK4/7), and eventually the ultimate substrate itself, MAPK (Fus3, ERK1/2, and JNK1/3). Recent studies, as well as our current one, have demonstrated that FAK may also act in a similar manner, as Yujiri et al. (48) have shown that MEK1 kinase can also directly associate with FAK. Thus it appears likely that many members of the canonical MAPK cascade can be organized by FAK at focal adhesion sites. This allows an extra level of specification of MAPK activation that would allow and promote the phosphorylation of focal adhesion complex proteins by ERK rather than nuclear substrates such as transcription factors. Such hypotheses on the determination of the targets for activated ERK have been described recently by Luttrell (49); hence the generation of ERK-activating scaffolds at epidermal growth factor receptor sites results in the generation of mitogenically active ERK protein, whereas ERK activation through Pyk2 scaffolds (similar in function to FAK) results in the generation of non-mitogenically active ERK protein (50). In agreement with our hypothesis the active ERK in rat embryo fibroblasts has been shown recently (51) to be localized to the same point in the focal adhesions where it participates in the regulation of cell adhesion and organization of the cytoskeletal network. Indeed when assessed for its mitogenic or proliferative action, we found that despite potently and chronically activating ERK, GnRH treatment of the SCL60 cells resulted in an anti-proliferative effect upon cell growth (data not shown) confirming the lack of “mitogenically” active ERK upon GnRH stimulation. Therefore, perhaps in a similar manner to β-arrestin (50), FAK can retain active ERK, constraining it to the cytoskeleton rather than allowing it to translocate to the nucleus. The requirement for ERK activation complex formation at the GnRH-activated FAK was further reinforced by the demonstration that overexpression of point mutant forms of FAK deficient in Src (52) or Grb2 binding (53) blunted the GnRH-induced activation of ERK (Fig. 8). We have demonstrated that there is a GnRH-induced cytoskeletal reorganization required for the FAK-mediated ERK-activating signaling complex assembly. This pathway was further delineated by demonstrating activation of the Rac-type monomeric Rho family G protein, presumably causing membrane remodeling (Fig. 2), directly preceded the enhanced tyrosine phosphorylation of FAK. Therefore, with increased cell spreading a greater magnitude of integrin engagement of ECM occurs resulting in the elevated tyrosine phosphorylation status of FAK (Fig. 3, panel b). When a dominant negative construct of Rac, but not Rho, was co-expressed with the HA-tagged wild-type FAK, we noted a significant inhibition of the GnRH-induced FAK phosphorylation (Fig. 9, panel a) and also a profound effect upon cell morphology. It appears likely that GnRH receptor activation of this member of the Rho family monomeric G protein gives rise to a profound plasma membrane remodeling that reinforces existing focal adhesions and also generates more sites of integrin attachment. In accordance with our implication of Rac in the GnRH-induced phosphorylation of FAK, the GnRH-stimulated remodeling of the cytoskeleton and plasma membrane formed many lamellipodia-like structures that are highly characteristic of Rac activation (Fig. 2, panels 4 and 6). The precise mechanism by which GPCRs can control Rac-like proteins remains relatively poorly understood. The activation of these

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**Fig. 10. Divergent signaling of the GnRH receptor is dependent upon active cytoskeletal remodeling.** The model represents the divergent signaling, through heterotrimeric and monomeric G proteins, from the mammalian type I GnRH receptor. The classical second messengers, inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG), are generated via a mechanism that can occur without cytoskeletal remodeling, whereas the activation of MAPK isoforms is limited to cell situations in which dynamic cell remodeling can occur. Upon receptor activation the monomeric G protein Rac causes profound ultrastructural changes of the plasma membrane resulting in enhanced association of αβ integrin dimers (red bars) with ECM proteins. Following the cytoskeletal changes, the activation of FAK results in the generation of an ERK1/2 activating signaling complex involving c-Src. PiP$_2$, phosphatidylinositol 4,5-bisphosphate.
monomeric proteins involves GDP-GTP exchange catalyzed usually by Dbl family Rac/Rho-GEFs, e.g. GEFT (54) or DOCK2 (55). The targeting to the plasma membrane of the Rho-GEF and indeed the monomeric G protein itself may occur via the Dbl homology or pleckstrin homology domains present on such Rho-GEFs as GEFT. Therefore, activation of a chronically signaling G protein-coupled receptor like the type I GnRH receptor would result in the generation of many free βγ subunits around the active receptor, which serve to attract the GEF to the membrane via its pleckstrin homology domain. Therefore, through the activation of the heterotrimeric Gαi, G protein, the release of its free βγ subunits may serve to induce the activation of the Rac family pathway. It is interesting, however, in the current study that the activation of the conventional PLC-β pathway, resulting in the Ca2+/PKC signaling system, can be distinguished from the MAPK activation pathway via the latter’s reliance upon the active cytoskeletal remodeling event (Fig. 2). As shown in Fig. 10, the generation of free inositol phosphates is not affected by cytochalasin D or latrunculin B, and conversely PLC-β inhibition does not inhibit MAPK activation. Therefore, signal divergence can occur rapidly from the activation of the primary receptor activation pathway, i.e. activation of Goαi. This divergence of signaling shows an important distinction for different transduction cascades based on the integrity of the cell cytoskeleton. This may demonstrate that for many intracellular cascades their “direction” of action is implicitly controlled by the cellular architecture, i.e. the coordinated transfer of signaling molecules between cytoskeletal elements generates the specificity of cellular signaling.

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