Am J Physiol Cell Physiol
279: C2019–C2027, 2000.

Requirement of cortical actin organization for bombesin, endothelin, and EGF receptor internalization

J. ADRIAN LUNN, HELEN WONG, ENRIQUE ROZENGURT, AND JOHN H. WALSH†
Department of Medicine, School of Medicine, Center for Ulcer Research and Education Digestive Diseases Research Center and Molecular Biology Institute, University of California, Los Angeles, California 90095

Received 7 April 2000; accepted in final form 29 June 2000

Lunn, J. Adrian, Helen Wong, Enrique Rozengurt, and John H. Walsh. Requirement of cortical actin organization for bombesin, endothelin, and EGF receptor internalization. *Am J Physiol Cell Physiol* 279: C2019–C2027, 2000.—The role of actin organization in occupancy-induced receptor internalization remains poorly defined. Here we report that treatment of mouse Swiss 3T3 cells with latrunculin A, a potent inhibitor of actin polymerization (including cortical actin), inhibited the internalization of the endogenous bombesin/gastrin-releasing peptide (GRP) receptor, as judged by uptake of ^{125}I-labeled GRP or fluorescent Cy3-labeled bombesin. In contrast, cells pretreated with cytochalasin D showed minimal inhibition of bombesin/GRP receptor internalization. Similarly, pretreatment of Swiss 3T3 cells with the potent Rho-kinase inhibitor HA-1077, at concentrations (10–20 μM) that abrogated bombesin-mediated stress fiber formation, did not significantly alter receptor-mediated internalization of ^{125}I-GRP. These results indicate that bombesin/GRP receptor internalization depends on latrunculin A-sensitive cortical actin rather than on rapidly turning over actin stress fibers that are disrupted by either cytochalasin D or HA-1077. The rates and total levels of internalization of the endogenously expressed endothelin A receptor and epidermal growth factor receptor were also markedly reduced by latrunculin A in Swiss 3T3 cells. The potency of latrunculin A for inhibiting G protein-coupled receptor endocytosis was comparable to that for reducing internalization of the epidermal growth factor tyrosine kinase receptor. We conclude that cortical actin structures, disrupted by latrunculin A, are necessary for occupancy-induced receptor internalization in animal cells.

signal transduction; heptahelical G protein coupled receptors; growth factor receptors; receptor cell biology; latrunculin; cytochalasin D; Rho kinase; Swiss 3T3 cells; epidermal growth factor; gastrin-releasing peptide

RECEPTOR-MEDIATED ENDOCYTOSIS plays a critical role in receptor downregulation, ligand degradation, and signal termination. Recent evidence has also implicated receptor internalization in signal transduction events critical for subsequent proliferation (12, 28), although this issue remains unresolved. Consequently, the mechanism(s) and regulation of agonist-promoted receptor internalization are attracting intense interest.

Genetic evidence in yeast has demonstrated a critical requirement for actin cytoskeleton in receptor-mediated endocytosis (1, 16, 23, 29). In contrast, the role of actin in receptor endocytosis in mammalian cells is less well defined (15). Experiments with polarized cells using cytochalasin D showed normal levels of receptor-mediated endocytosis at the basolateral surface while apical receptor-mediated uptake was inhibited (18, 22). Using nonpolarized A431 cells and several agents that bind monomeric actin to promote actin depolymerization including the cell-permeant macrolide latrunculin A, Lamaze et al. (26) showed a requirement for cortical actin cytoskeleton in transferrin receptor sequestration into coated pits. Transferrin receptors are known to undergo constitutive internalization and recycling (17). In contrast, the internalization of receptors for neuropeptides and growth factors is dramatically enhanced by agonist occupancy. Despite its potential importance, the role of cortical actin organization in agonist-dependent receptor internalization has not been defined.

Bombesin and its mammalian homolog, gastrin-releasing peptide (GRP), bind to a G protein-coupled receptor (GPCR) (2, 43) that activates mitogenic signal transduction pathways via the heterotrimeric G proteins G_q and G_{12} (35, 36) and promotes rapid and extensive receptor internalization (19, 44). In the present study, we demonstrate that treatment with latrunculin A inhibits the rate and extent of ligand-induced internalization via the endogenously expressed bombesin/GRP receptor in Swiss 3T3 cells. Exposure to latrunculin A also reduced the internalization of endothelin (ET) A receptor, a different GPCR, and of the tyrosine kinase receptor for epidermal growth factor (EGF). Our results show that latrunculin A inhibited receptor internalization much more effectively than cytochalasin D, suggesting that cortical actin organization plays a critical role in agonist-dependent receptor internalization in animal cells.

†Deceased 14 June 2000.

Address for reprint requests and other correspondence: E. Rozengurt, Rm. 115, Bldg. 115, CURE Digestive Diseases Research Center, GLAVAHS, 11301 Wilshire Blvd., Los Angeles, CA 90073-1792 (E-mail: erozengurt@mednet.ucla.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

MATERIALS AND METHODS

Materials. Latrunculin A was obtained from Molecular Probes, Eugene, OR. Cytochalasin D, rhodamine-phalloidin, GRP, bombesin, ET, EGF, and FBS were obtained from Sigma. The Rho-kinase inhibitor 1-(5-isoquinolinesulfonyl)-homopiperazine (HA-1077) was obtained from Calbiochem, San Diego, CA. ^{125}I-labeled GRP (2,000 Ci/mmol) and Cy3 were purchased from Amersham. Labeling of bombesin-peptide solution with Cy3 dye was done as per catalog protocol in 0.1 M sodium carbonate buffer for 30 min at room temperature. Labeled peptide was separated from unlabeled with HPLC. ^{125}I labeling of peptides was done using the soluble lactoperoxidase method, and unlabeled peptide was separated using HPLC.

Cell culture. Stock cultures of Swiss 3T3 cells were maintained in DMEM containing 10% FBS penicillin (100 U/ml), and streptomycin (100 μg/ml) in humidified 10% CO_2-90% air at 37°C. For experimental purposes cells were subcultured into 35-mm Nunc dishes (10^5 cells/dish) or 100-mm Nunc dishes (6×10^5 cells/dish) in DMEM containing 10% fetal bovine serum (FBS). After 5–7 days, the cultures were confluent and quiescent as shown by autoradiography (<1% labeled nuclei) after 40-h exposure to [^{3}H]thymidine and flow cytofluorometric analysis (13).

Pretreatment conditions. Swiss 3T3 cells were plated on Nunc 24-well plates at 5×10^5 cells/well in DMEM with 10% FBS. Overnight cultures were washed with DMEM and then pretreated with latrunculin A (9 μM), cytochalasin D (1 μM), sucrose (0.45 M), or HA-1077 (10 or 20 μM), all in DMEM. The cultures were exposed to these agents for the times indicated in the legends to Figs. 1–7.

Receptor internalization assay. Labeled peptides were added to cells in triplicates with 1,000-fold excess cold peptide added to one of the wells to determine specific binding. The cells were then incubated for various times at 37°C. After specified time points expired, the medium was rapidly removed and the cultures were washed with ice-cold PBS solution containing 0.1% BSA. Then 1 ml of ice-cold acid wash (0.2 M acetic acid and 0.5 M NaCl) was added to the cultures for 5 min. The acid-extractable fraction was collected by aspiration, and the acid-resistant fraction was collected after solubilization of the cell monolayer with lysis buffer (2% $NaHCO_3$, 1% SDS, and 0.1 M NaOH). Both acid wash and cell-associated radioactivity were counted in a gamma counter.

Fluorescent Cy3-bombesin imaging. Cultures of Swiss 3T3 cells were grown to quiescence, as described above. Cy3-bombesin was added in DMEM/Waymouth medium MB752/l supplemented with *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) and NaOH to pH 7.4 and 0.1% BSA (fraction V Sigma) at 37°C for 10 min. Cells were imaged immediately using a Ziess Axioskope 2 microscope and a ×100 water objective. Images were obtained using a Spot digital camera and associated software (Spot, Image Diagnostics) and further processed using Adobe Photoshop.

Visualization of actin organization. Swiss 3T3 cells were grown as above, on 35-mm plastic Nunc dishes to quiescence. After pretreatments, the cells were fixed for 1 h in 4% paraformaldehyde in PBS pH 7.4. They were washed twice with PBS then permeabilized using 0.2% Triton X-100 for 10 min. The fixed and permeabilized cells were then exposed to a blocking solution (DMEM/Waymouth medium MB752/l supplemented with BES and NaOH to pH of 7.4, 0.1% BSA, and 2% FBS) for 30 min at room temperature. Rhodamine-phalloidin (Sigma), diluted in the blocking solution to 1 μg/ml, was added to the cells for 30 min at room temperature. The stained cells were washed with PBS six times. They were then either imaged directly, with a Ziess Axiophot microscope using a ×100 water objective, or dried and covered with Vectashield H100 and VWR no. 1 coverslips and imaged with the same microscope using a ×63 oil-immersion objective. Images were obtained using a Spot camera and associated software (Spot, Image Diagnostics) and further processed using Adobe Photoshop.

Intracellular Ca^{2+} concentration measurements. Swiss 3T3 cells (4×10^5 cells/ml) were grown on poly-L-lysine-coated glass coverslips (Hitachi) in 35-mm dishes overnight. Cells were washed twice with buffer A (Hanks' balanced salt solution supplemented with 4 mM Na_2HCO_3, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, and 0.1% BSA) and incubated at room temperature in 1 ml of buffer A containing 2.5 μM fura 2-AM ester. The cells were then washed four times with buffer A and incubated at 25°C for 30 min in buffer A containing either latrunculin A, or an equivalent amount of solvent (control). The coverslips with fura-2-loaded cells were then placed into a quartz cuvette inserted into a Hitachi model F-2000 spectrofluorometer. The excitation wavelengths were at 340 and 380 nm, and the emission wavelength was set at 510 nm. Intracellular calcium concentration ($[Ca^{2+}]_i$) was calculated using the formula $[Ca^{2+}]_i$ (in nM) = $K(F - F_{min})/(F_{max} - F)$, where F is the fluorescence at the unknown $[Ca^{2+}]_i$, F_{max} was determined by injecting 20–40 μl of 5 mM digitonin into the cuvette, and the F_{min} was measured after injection of 40 μl of 0.5 M EDTA, pH 8.0. The value of K for fura 2 used was 224 nM (20).

RESULTS

The binding of bombesin/GRP to its heptahelical GPCR induces phosphorylation (24, 25) and rapid internalization via clathrin-coated pits (19, 44) of this receptor. In the present study, we monitored receptor-mediated bombesin/GRP internalization by using two different techniques. In the first method, cultures of Swiss 3T3 cells were transferred to binding medium at 37°C for 1 h and then exposed to ^{125}I-GRP. At various times of incubation, the cultures were rapidly washed to remove unbound radioactive ligand and subjected to acid-salt extraction to selectively dissociate surface-bound ligand. Acid-extractable (equivalent to surface-bound ligand) and acid-resistant (corresponding to receptor-mediated internalized ligand) radioactivity were then measured. Figure 1*A* shows that acid-resistant ^{125}I-GRP accumulated linearly up to 15 min of incubation into Swiss 3T3 cells.

In the second method, Swiss 3T3 cells, equilibrated in binding solution for 1 h, were incubated with fluorescent Cy3-labeled bombesin. After 10 min at 37°C, the cultures were rapidly washed, and fluorescence accumulated into intact cells was visualized using a Ziess Axioskope 2 microscope with a ×100 water objective lens. Figure 1*B* shows a representative cell in which Cy3-bombesin was internalized into vesicles (most likely endosomes). The results presented in Fig. 1, *A* and *B*, confirm that the bombesin/GRP receptor mediates rapid and extensive ligand internalization into Swiss 3T3 cells.

Effect of latrunculin A and cytochalasin D on bombesin/GRP receptor internalization. To determine whether actin organization is required for the internalization of

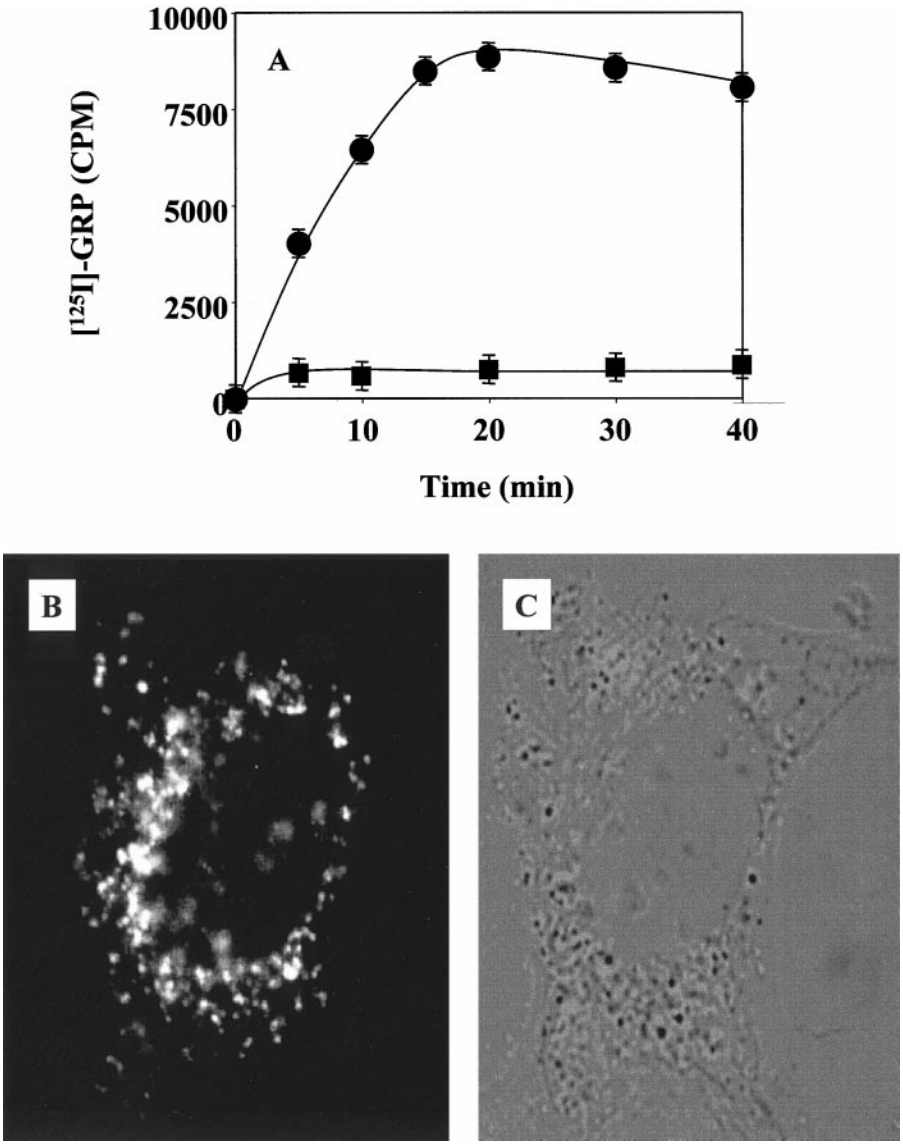

Fig. 1. Receptor-mediated gastrin-releasing peptide (GRP) internalization by Swiss 3T3 cells. *A*: quiescent cultures of Swiss 3T3 cells were incubated at 37°C for various times with 0.5 ml of DMEM containing 2 nM GRP and 400,000 counts/min (cpm) of ^{125}I-GRP. Cells were washed in ice-cold PBS, and then surface and internalized ^{125}I-GRP were determined by subjecting the cells to acid wash (surface) followed by solubilization in lysis buffer (internal), as described in MATERIALS AND METHODS. Each time point was evaluated in sets of three wells, with the third well containing a 1,000-fold excess of unlabeled GRP for determination of nonspecific binding. Squares represent surface-bound ^{125}I-GRP, and the circles represent internalized ^{125}I-GRP. Each value represents the mean ± SE from duplicate samples from at least 3 separate experiments. *B*: Swiss 3T3 cells were incubated with Cy3-labeled bombesin for 30 min at 37°C, at which time they were rapidly washed with PBS at 37°C and visualized using an inverted Ziess microscope and the ×100 aqueous objective lens. The fluorescence micrograph shows a typical endosomal uptake pattern. *C*: shows same cell as in *B* with Nomarski differential-interference contrast optics using the same lens and microscope.

the GPCR for bombesin/GRP, we utilized the agents cytochalasin D and latrunculin A, which induce actin cytoskeleton depolymerization through different mechanisms (3). Cytochalasin D binds to the growing end of actin filaments, leading to disruption of actively turning over actin stress fibers (11). Latrunculin (40) sequesters actin monomers and effectively disrupts both actin stress fibers and cortical actin filaments (40), which are more resistant to cytochalasin D (8). Visualization of the actin cytoskeleton with rhodamine-phalloidin staining of fixed cells after various times of exposure to either 9 μM latrunculin A (0–60 min) or 2 μM cytochalasin D (0–120 min) was performed to assess the kinetics and extent of cytoskeletal disruption. Maximal disruption of actin cytoskeleton in Swiss 3T3 cells by latrunculin A or cytochalasin D occurred after 30 or 120 min of incubation, respectively (data not shown). Consequently, the cells were pretreated for 1 h with latrunculin A or for 2 h with cytochalasin D in all subsequent internalization assays in which the effects of these agents were tested.

To assess the effect of latrunculin A and cytochalasin D on bombesin/GRP receptor internalization, quiescent cultures of Swiss 3T3 cells were washed and transferred to media containing these agents or solvent control. Then the cultures were incubated with ^{125}I-GRP for 10 min, washed to remove unbound radioactive ligand, and subjected to an acid-salt extraction procedure to selectively dissociate surface-bound ligand. Acid-extractable (equivalent to surface-bound ligand) and acid-resistant (corresponding to receptor-mediated internalized ligand) radioactivity were then measured.

As shown in Fig. 2*A*, ~65% of specific cell-associated ^{125}I-GRP was internalized (i.e., acid resistant) in control cells after 10 min of incubation. Preincubation with cytochalasin D caused a small but statistically significant decrease in the extent of ^{125}I-GRP internalization. In contrast, prior exposure to latrunculin A caused a marked decrease in the level of ^{125}I-GRP internalized in Swiss 3T3 cells and produced a concom-

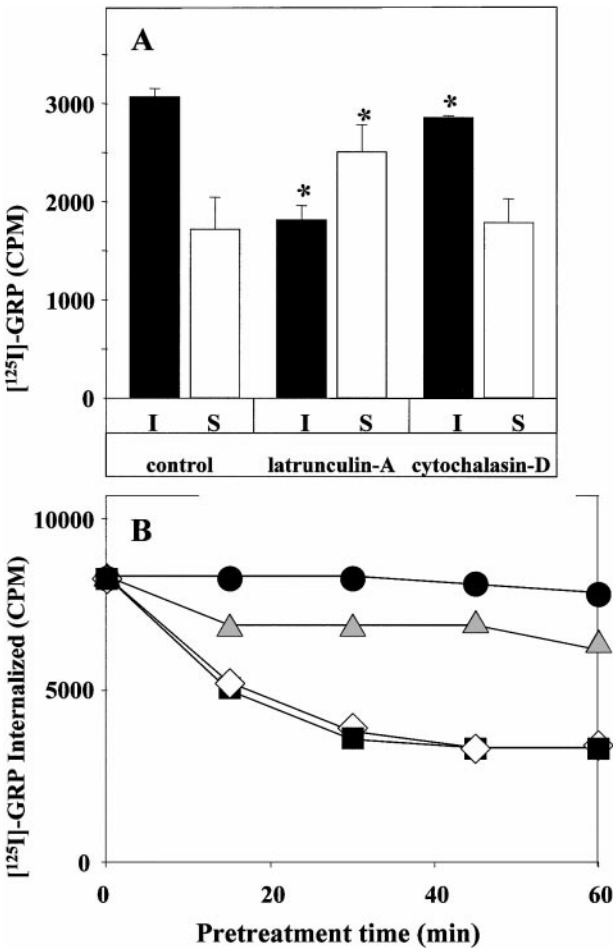

Fig. 2. Effect of latrunculin A on internalization of bombesin/GRP receptors. *A*: quiescent Swiss 3T3 cells were pretreated at 37°C for 1 h with or without 9 μM latrunculin A in DMEM or for 2 h in DMEM containing 2 μM cytochalasin D as indicated. Then, 2 nM GRP containing 400,000 cpm of ^{125}I-GRP was added to each culture well, and they were incubated for a further 10 min at 37°C. After washing with ice-cold PBS containing 0.1% BSA, surface (S, open bars) and internalized ^{125}I-GRP (I, solid bars) were determined by subjecting the cells to acid-salt extraction (surface) followed by solubilization of remaining radioactivity in lysis buffer (internal), as described in MATERIALS AND METHODS. Bars are means ± SE of 8 independent determinations. *B*: quiescent Swiss 3T3 cells were pretreated at 37°C, for various times in DMEM alone or in this medium containing 2 μM cytochalasin D, 9 μM latrunculin A, or 0.45 M sucrose. At various times after pretreatment initiation, 2 nM GRP containing 400,000 cpm of ^{125}I-GRP was added to these pretreated cells and the internalized counts were assayed as in *A* after a 10-min incubation. The graph shows internalized cpm after pretreatment with binding DMEM alone (circles), 2 μM cytochalasin D (triangles), 9 μM latrunculin A (squares), or 0.45 M sucrose (diamonds).

itant increase in the level of surface-bound ligand ($P < 0.0005$).

The inhibition of receptor-mediated ^{125}I-GRP internalization was a rapid consequence of cell exposure to latrunculin A. Maximal inhibition of receptor internalization occurred after 30 min of incubation of the cells with latrunculin A (Fig. 2*B*). The results shown in Fig. 2*B* also indicate that treatment with 9 μM latrunculin A was as effective as exposure to medium containing 0.45 M sucrose (a well-known disrupter of clathrin-coated pit formation) in preventing ^{125}I-GRP internalization. These results indicate that latrunculin A prevents internalization of the bombesin/GRP receptor in Swiss 3T3 cells.

We also examined the effect of exposure to latrunculin A or cytochalasin D on bombesin/GRP receptor-mediated endocytosis using Cy3-labeled bombesin. As shown in Fig. 3, treatment of Swiss 3T3 cells with latrunculin A (9 μM, 1 h) greatly diminished the uptake of Cy3-bombesin into intracellular vesicles, whereas treatment with cytochalasin D (2 μM, 120 min) only slightly decreased the uptake of Cy3-bombesin (Fig. 3, *A*, *C*, and *E*). As expected, both agents induced rounding of Swiss 3T3 cells, consistent with their ability to completely disrupt actin stress fibers (Fig. 3, *B*, *D*, and *F*).

Kinetics of ^{125}I-GRP and ^{125}I-bombesin internalization. Next, we determined the time course of ^{125}I-GRP internalization in Swiss 3T3 cells incubated in the

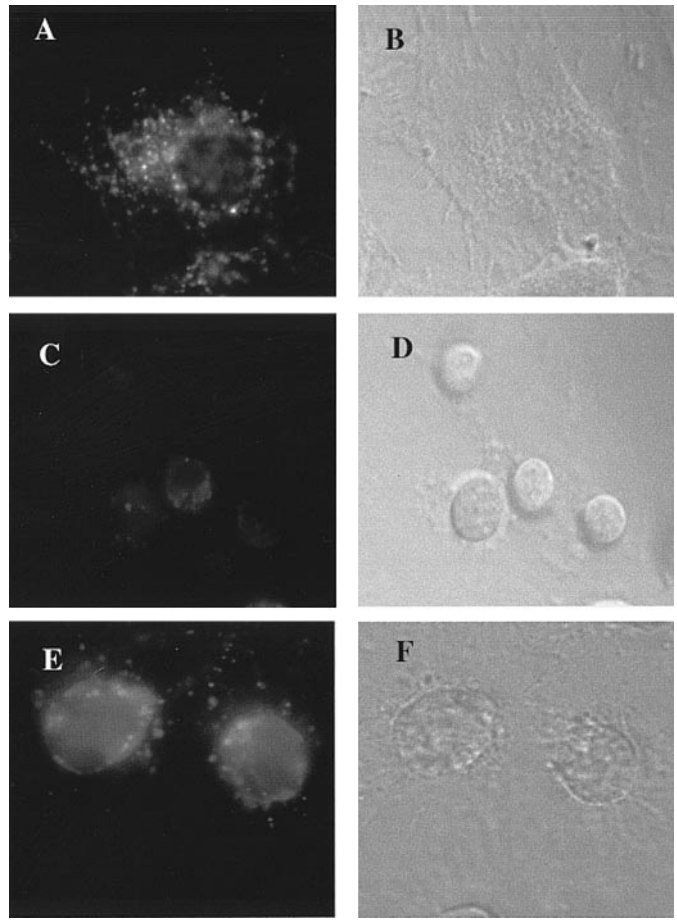

Fig. 3. Visualization of Cy3-bombesin uptake into live Swiss 3T3 cells in the absence or presence of latrunculin A. Quiescent Swiss 3T3 cells were pretreated for 1 h in binding buffer alone (*A* and *B*), in binding buffer containing 9 μM latrunculin A (*C* and *D*), or in binding buffer containing 2 μM cytochalasin D (*E* and *F*). Pretreated cells were then incubated with Cy3-labeled bombesin for 10 min at 37°C, at which time they were rapidly washed with PBS and visualized using a Zeiss Axioscope 2 microscope and ×100 aqueous objective lens. Light micrographs in *B*, *D*, and *F* show the same fields of cells as in *A*, *C*, and *E*, respectively, using Nomarski differential-interference contrast optics.

absence or in the presence of either cytochalasin D or latrunculin A. In control cultures, acid-resistant ^{125}I-GRP increased rapidly reaching a maximal level 15 min after addition of the labeled peptide. Pretreatment with cytochalasin D resulted in a small decrease in the rate of ^{125}I-GRP internalization, but it did not affect the total amount of peptide internalized. In contrast, prior exposure to latrunculin A caused a dramatic decrease in the rate of ^{125}I-GRP internalization and in the total amount of peptide internalized even after 40 min of incubation (Fig. 4*A*).

To substantiate the differential effects of latrunculin and cytochalasin D obtained with ^{125}I-GRP, we also determined the rate of receptor internalization using ^{125}I-bombesin, a labeled agonist that binds to the same GPCR. In agreement with the results obtained with ^{125}I-GRP, addition of cytochalasin D caused a small decrease in the rate of ^{125}I-bombesin internalization, whereas addition of latrunculin A caused striking inhibition of ^{125}I-bombesin internalization.

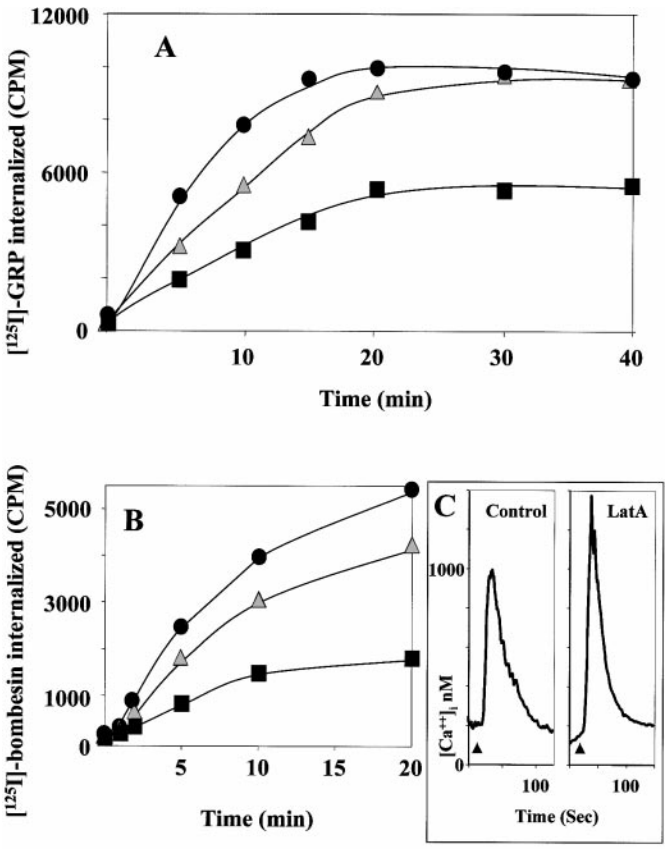

Fig. 4. Effect of latrunculin A and cytochalasin D on the kinetics of ^{125}I-GRP and ^{125}I-bombesin internalization in Swiss 3T3 cells. Quiescent Swiss 3T3 cells were pretreated at 37°C for 1 h in binding buffer (circles) 1 h in binding buffer containing 9 μM latrunculin A (squares), or 2 h in binding buffer containing 2 μM cytochalasin D (triangles). After these pretreatments, 2 nM GRP (*A*) or 1 nM bombesin (*B*), each containing ~400,000 cpm of the corresponding ^{125}I-labeled peptide, was added, and the cells were incubated at 37°C for various times, as indicated. Internalized radioactivity was determined at various times by selective removal of surface label by acid-salt extraction followed by solubilization with lysis buffer. Each point represents the mean of duplicate determinations. *C*: latrunculin A (Lat A) does not affect Ca^{2+} mobilization in response to bombesin. Swiss 3T3 cells, previously plated on coverslips, were loaded with fura-2 and then incubated for 30 min with either buffer alone or with buffer containing 9 μM latrunculin A. The effect of 1 nM bombesin on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined as described in MATERIALS AND METHODS. The addition of bombesin is indicated by the arrowheads.

Bombesin-mediated Ca^{2+} transients in Swiss 3T3 cells are not affected by pretreatment with latrunculin A. One of the earliest responses induced by bombesin binding to its receptor is the $G\alpha_q$-mediated, phospholipase C-β-catalyzed production of inositol 1,4,5-trisphosphate, which triggers release of Ca^{2+} from internal stores. To examine whether treatment with latrunculin A interferes with early signaling by the bombesin/GRP receptor, we measured the transient increase in $[Ca^{2+}]_i$, stimulated by bombesin in Swiss 3T3 cells pretreated in the absence or presence of latrunculin A. Figure 4*C* shows a typical tracing of $[Ca^{2+}]_i$ from fura 2-loaded Swiss 3T3 cells in response to 1 nM bombesin stimulation. Treatment with latrunculin A had no apparent effect on the transient increase in $[Ca^{2+}]_i$ stimulated by bombesin in Swiss 3T3 cells, in agreement with recent results reported by Patterson et. al. (31). Hence, despite dramatic morphological changes induced by latrunculin A, cells treated with this agent remained viable because early phospholipid-dependent pathways downstream of the bombesin/GRP receptor were still intact.

Effect of HA-1077 on GRP internalization. The bombesin/GRP GPCR interacts with members of the G_{12} family of GPCRs, leading to Rho-dependent formation of actin stress fibers, assembly of focal adhesion plaques, and tyrosine phosphorylation of focal adhesion proteins (4, 9, 30, 33, 34, 38, 45). A major downstream effector of Rho leading to cytoskeletal responses is the serine/threonine protein kinase ROK that is activated by Rho-GTP (21). Given the above results suggesting that ligand-dependent internalization of the bombesin/GRP receptor depends on cortical actin organization, we examined whether inhibition of ROK activity could alter bombesin/GRP receptor internalization. As shown in Fig. 5, pretreatment of Swiss 3T3 cells with the ROK inhibitor HA-1077 (32, 37), at concentrations (10–20 μM) that abrogated bombesin-mediated stress fiber formation (Fig. 5, *A–D*), did not significantly alter the internalization of ^{125}I-GRP (Fig. 5, *bottom*). The preceding findings support the conclusion that GPCR internalization depends on latrunculin A-sensitive cortical actin rather than on actively turning over actin stress fibers.

Latrunculin A inhibits GPCR-mediated ^{125}I-ET internalization. The preceding results indicate that disruption of cortical actin organization by latrunculin A inhibits ligand-induced internalization of the bombesin/GRP receptor in Swiss 3T3 cells. We examined whether the cortical actin cytoskeleton is also required for the internalization of the ET receptor subtype A, a different GPCR that, like the bombesin/GRP receptor, is endogenously expressed by Swiss 3T3 cells (14). As shown in Fig. 6*A*, acid-resistant ^{125}I-ET increased in a time-dependent manner in control cells. Treatment

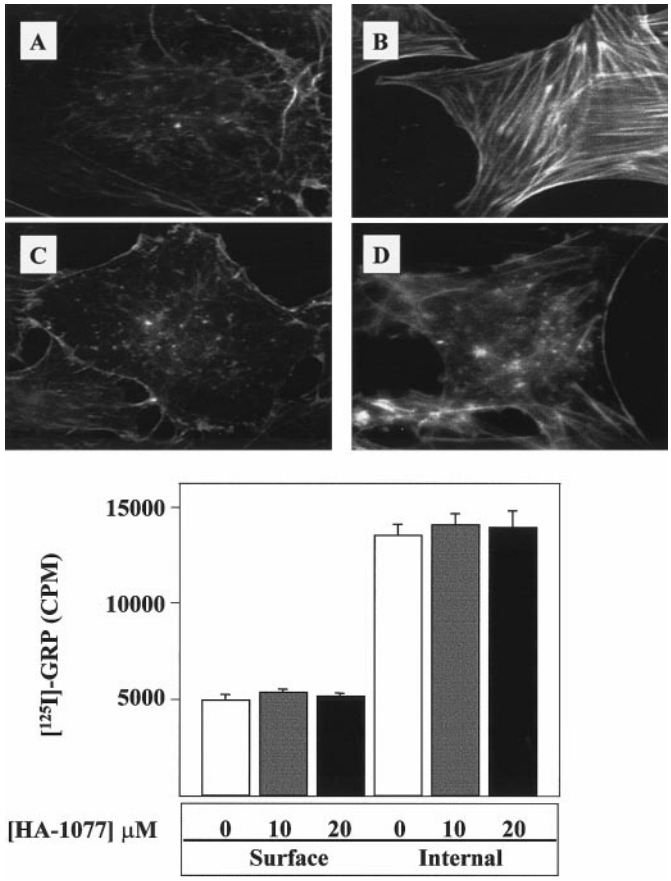

Fig. 5. The Rho-kinase inhibitor HA-1077 prevents bombesin-stimulated actin remodeling but does not affect GRP receptor internalization. *Top*: quiescent Swiss 3T3 cells were pretreated for 30 min at 37°C with binding buffer (*A* and *B*) or binding buffer containing 20 μM HA-1077 (*C* and *D*). Bombesin, 1 nM, was added to some of the cultures (*B* and *D*). After 15 min of incubation, the cells were washed, fixed, and stained with rhodamine-phalloidin. *Bottom*: effect of prior exposure for 30 min to HA-1077 at either 10 μM or 20 μM on surface and internalized ^{125}I-GRP. Swiss 3T3 cells treated with or without HA-1077 were incubated with labeled peptide for 10 min at 37°C. Values are means ± SE from triplicate samples from at least 2 separate experiments.

with latrunculin A markedly reduced the rate and the total amount of ^{125}I-ET internalization. These results indicate that the requirement for latrunculin A-sensitive cortical actin cytoskeleton is not restricted to the internalization of the bombesin/GRP receptor.

Latrunculin A inhibits EGF receptor-mediated ^{125}I-EGF internalization. Activation of the EGF receptor (EGFR) by EGF is known to induce rapid clathrin-dependent internalization of ligand-receptor complexes and the subsequent degradation of both the growth factor and the receptor in lysosomes (6, 27, 39, 42). Internalization-defective EGFR mutants are more efficient in transducing mitogenic and transforming signals (10, 41). Despite its potential importance, the influence of cortical actin organization on EGFR internalization has not been defined. As shown in Fig. 6 *B*, the rapid increase in acid-resistant ^{125}I-EGF in Swiss 3T3 cells was markedly reduced by prior exposure to latrunculin A.

The results shown in the *insets* of Fig. 6, *A* and *B*, indicate that treatment with 9 μM latrunculin A was as effective as exposure to medium containing 0.45 M sucrose (a well-known disrupter of clathrin-coated pit formation) in preventing either ^{125}I-ET or ^{125}I-EGF

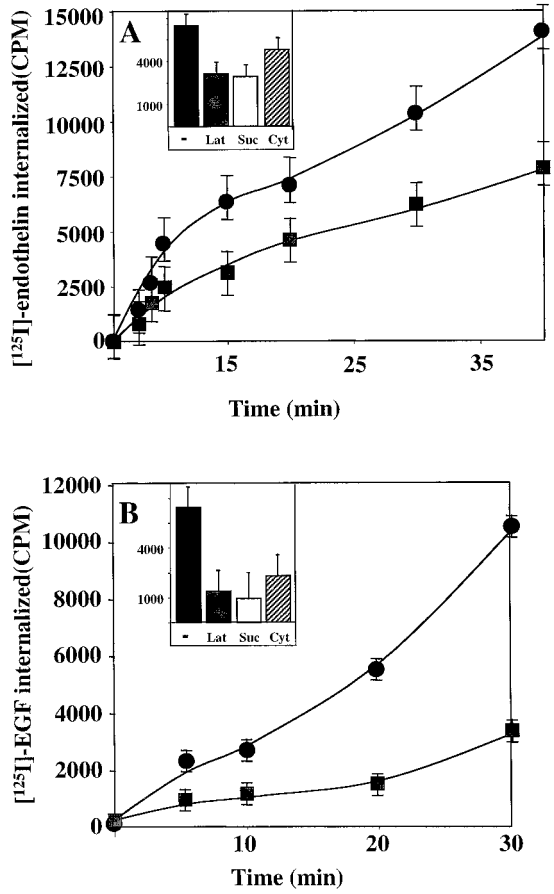

Fig. 6. Treatment with latrunculin A prevents receptor-mediated internalization of ^{125}I-endothelin (^{125}I-ET) and ^{125}I-labeled epidermal growth factor (^{125}I-EGF). *A*: quiescent Swiss 3T3 cells were pretreated for 1 h in binding buffer in the absence (circles) or in the presence of 9 μM latrunculin A (squares). ^{125}I-ET was then added for various times at 37°C. After washing rapidly with cold PBS containing 0.1% BSA, internalized radioactivity was determined after acid-salt extraction, as described in MATERIALS AND METHODS. *B*: quiescent Swiss 3T3 cells were pretreated for 1 h in binding buffer in the absence (circles) or in the presence of 9 μM latrunculin A (squares). ^{125}I-labeled EGF was then added for various times at 37°C. After washing rapidly with cold PBS containing 0.1% BSA, internalized radioactivity was determined after acid-salt extraction, as described in MATERIALS AND METHODS. *Inset* of *A*: quiescent Swiss 3T3 cells were pretreated for 1 h in binding buffer (−, solid bar), in binding buffer with 9 μM latrunculin A (Lat, gray bar), in binding buffer with 0.45 M sucrose (Suc, open bar), or for 2 h in binding buffer with 2 μM cytochalasin D (Cyt, hatched bar). ^{125}I-ET was then added for 10 min at 37°C. After washing rapidly with cold PBS containing 0.1% BSA, internalized radioactivity was determined after acid-salt extraction, as described in MATERIALS AND METHODS. *Inset* for *B*: quiescent Swiss 3T3 cells were pretreated for 1 h in binding buffer (−, solid bar), in binding buffer with 9 μM latrunculin A (Lat, gray bar), in binding buffer with 0.45 M sucrose (Suc, open bar), or for 2 h in binding buffer with 2 μM cytochalasin D (Cyt, hatched bar). ^{125}I-labeled EGF was then added for 20 min at 37°C. After washing rapidly with cold PBS containing 0.1% BSA, internalized radioactivity was determined after acid-salt extraction, as described in MATERIALS AND METHODS. Values are means ± SE of 3 independent experiments.

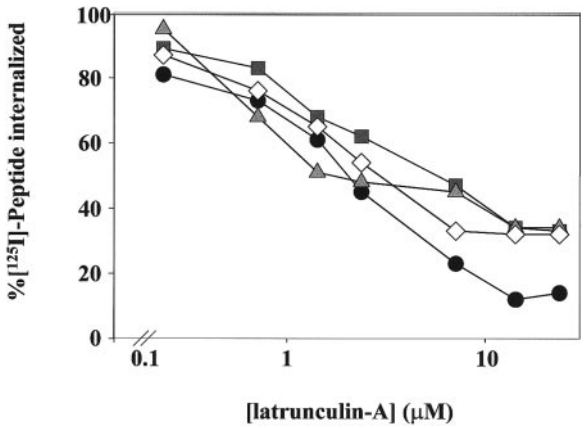

Fig. 7. Latrunculin A pretreatment inhibits receptor-mediated internalization of bombesin, GRP, ET, and EGF in a dose-dependent manner. The internalization of ^{125}I-labeled bombesin (circles), GRP (squares), ET (triangles), and EGF (diamonds) was assessed in quiescent Swiss 3T3 cells pretreated for 1 h with increasing concentrations of latrunculin A. Internalized radioactivity was determined after acid-salt extraction for 5 min, as described in MATERIALS AND METHODS. The graph shows a plot of internalized labeled peptide as a function of latrunculin A concentration (expressed as a percentage of the radioactivity internalized by cells not exposed to latrunculin A). All assays of internalized peptide were performed after 10 min of incubation with labeled peptide. Each point represents the mean of duplicate determinations.

internalization. Treatment with cytochalasin D demonstrated intermediate efficacy in preventing either ^{125}I-ET or ^{125}I-EGF internalization. These findings indicate that cortical actin organization is required for the internalization of both GPCR and tyrosine kinase receptors.

Effect of increasing concentrations of latrunculin A on the internalization of GPCRs and EGFR. We compared the potency of latrunculin A for inhibiting the internalization of the GPCRs for bombesin/GRP and ET with that of the tyrosine kinase receptor for EGF, all of which are endogenously expressed in Swiss 3T3 cells. Cultures of these cells were exposed to increasing concentrations of latrunculin A for 1 h and then incubated with ^{125}I-GRP, ^{125}I-ET, or ^{125}I-EGF for 10 min, and acid-resistant radioactivity was measured in each case to determine receptor-mediated ligand internalization. As illustrated by Fig. 7, latrunculin A inhibited receptor-mediated internalization of these ligands in a very similar dose-dependent fashion. These results imply that the internalization of all three receptors requires an intact actin cortical cytoskeleton.

DISCUSSION

Recent evidence indicates the existence of an intricate interplay between the multistep process of receptor internalization and the organization of the cortical actin cytoskeleton. Genetic evidence in yeast has demonstrated an important role for the actin cytoskeleton in receptor-mediated endocytosis (1, 16, 23, 29), and agents that bind to monomeric actin leading to the disruption of the cortical actin cytoskeleton have been shown to inhibit transferrin receptor internalization in mammalian cells (26). Transferrin receptors are known to undergo constitutive internalization and recycling (17), whereas the receptors for neuropeptides and growth factors require agonist binding for internalization. The role of cortical actin organization in agonist-dependent receptor internalization has not been investigated. These considerations assume an added interest in view of the putative role of GPCR internalization in signal transduction (12, 28).

The experiments presented here were designed to clarify the requirement of actin cytoskeleton organization and the role of Rho-dependent actin remodeling in the endocytosis of endogenously expressed GPCRs in Swiss 3T3 cells. In this study we examined the effect of cytochalasin D and latrunculin A on the endocytosis of the bombesin/GRP receptor, a receptor known to internalize through clathrin-coated pits. Cytochalasin D caps the growing end of actin filaments and thus disrupts actively turning over actin stress fibers but appears to have much less effect on cortical actin (11). In contrast, latrunculin A binds to G-actin, forming a nonpolymerizable 1:1 molar complex (40), and thus it disrupts both stress fibers and cortical actin, which are more resistant to cytochalasin D (8)

Our results show that exposure of Swiss 3T3 cells to latrunculin A profoundly inhibits the internalization of the bombesin/GRP receptor. In contrast, treatment with cytochalasin D reduced only slightly the rate of receptor-mediated endocytosis, and it did not affect the total level of internalized ligand. The differential effects of latrunculin A and cytochalasin D imply that bombesin/GRP receptor internalization is not affected by disruption of rapidly turning over actin stress fibers. In support of this conclusion, we demonstrate that inhibition with HA-1077 of ROK, a downstream effector of Rho that plays a key role in GPCR-induced actin stress fiber formation, did not affect bombesin/GRP receptor internalization. These findings support the conclusion that receptor-mediated internalization of bombesin/GRP depends on latrunculin A-sensitive cortical actin rather than on actively turning over actin stress fibers.

The inhibitory effect of latrunculin A on receptor internalization is not confined to the bombesin/GRP receptor, since we found that the rate and total amount of ^{125}I-ET internalization via the endogenously expressed ET_A receptor are also markedly reduced by latrunculin A in Swiss 3T3 cells. Furthermore, latrunculin A-mediated disruption of cortical actin organization also inhibited internalization of the tyrosine kinase EGFR, which also proceeds through clathrin-coated pits. The potency of latrunculin A for inhibiting GPCR-mediated receptor endocytosis was similar to that found for inhibition of EGFR internalization. Thus our results demonstrate that agonist-dependent internalization of either GPCRs or EGF tyrosine kinase receptor requires an intact cortical actin cytoskeleton. It is plausible that the latrunculin A-sensitive cortical actin is necessary for maintaining the spatial organization of proteins required for receptor-mediated endocytosis. Recently, the cortical cytoskeleton has also

been implicated in the sorting of GPCRs after internalization, identifying another step of receptor recycling that is dependent on the actin cytoskeleton (5).

Receptor internalization has been traditionally thought to play a role in receptor downregulation and signal termination, but recent evidence has implicated receptor endocytosis in signal transduction events critical for subsequent proliferation (12, 28). Previous results demonstrated that cytochalasin D is a potent inhibitor of tyrosine phosphorylation cascades stimulated by bombesin in Swiss 3T3 cells (7, 38, 46), indicating that these events depend on the formation of actin stress fibers. The differential effect of latrunculin and cytochalasin D on GPCR-mediated endocytosis uncovered by the results presented here offers a novel approach to dissect the role of cortical actin and receptor internalization in GPCR signal transduction.

This work was supported by National Institutes of Health Grants DK-37540, DK-41301, DK-17294, and DK-55003 and by Veterans Administration Research Funds.

REFERENCES

1. **Bâenâedetti H, Raths S, Crausaz F, and Riezman H.** The END3 gene encodes a protein that is required for the internalization step of endocytosis and for actin cytoskeleton organization in yeast. *Mol Biol Cell* 5: 1023–1037, 1994.
2. **Battey JF, Way JM, Corjay MH, Shapira H, Kusano K, Harkins R, Wu WJM, Slattery T, Mann E, and Feldman RI.** Molecular cloning of the bombesin/gastrin-releasing peptide receptor from Swiss 3T3 cells. *Proc Natl Acad Sci USA* 88: 395–399, 1991.
3. **Belmont LD, Patterson GM, and Drubin DG.** New actin mutants allow further characterization of the nucleotide binding cleft and drug binding sites. *J Cell Sci* 112: 1325–1336, 1999.
4. **Buhl AM, Johnson NL, Dhanasekaran N, and Johnson GL.** $G\alpha_{12}$ and $G\alpha_{13}$ stimulate Rho-dependent stress fiber formation and focal adhesion assembly. *J Biol Chem* 270: 24631–24634, 1995.
5. **Cao TT, Deacon HW, Reczek D, Bretscher A, and von Zastrow M.** A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β_2-adrenergic receptor. *Nature* 401: 286–290, 1999.
6. **Carter RE and Sorkin A.** Endocytosis of functional epidermal growth factor receptor-green fluorescent protein chimera. *J Biol Chem* 273: 35000–35007, 1998.
7. **Casamassima A and Rozengurt E.** Tyrosine phosphorylation of p130(cas) by bombesin, lysophosphatidic acid, phorbol esters, and platelet-derived growth factor. Signaling pathways and formation of a p130(cas)-Crk complex. *J Biol Chem* 272: 9363–9370, 1997.
8. **Cassimeris L, McNeill H, and Zigmond SH.** Chemoattractant-stimulated polymorphonuclear leukocytes contain two populations of actin filaments that differ in their spatial distributions and relative stabilities. *J Cell Biol* 110: 1067–1075, 1990.
9. **Charlesworth A, Broad S, and Rozengurt E.** The bombesin/GRP receptor transfected into Rat-1 fibroblasts couples to phospholipase C activation, tyrosine phosphorylation of p125FAK and paxillin and cell proliferation. *Oncogene* 12: 1337–1345, 1996.
10. **Chen WS, Lazar CS, Lund KA, Welsh JB, Chang CP, Walton GM, Der CJ, Wiley HS, Gill GN, and Rosenfeld MG.** Functional independence of the epidermal growth factor receptor from a domain required for ligand-induced internalization and calcium regulation. *Cell* 59: 33–43, 1989.
11. **Cooper JA.** Effects of cytochalasin and phalloidin on actin. *J Cell Biol* 105: 1473–1478, 1987.
12. **Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SS, Caron MG, and Lefkowitz RJ.** Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J Biol Chem* 273: 685–688, 1998.
13. **Dicker P and Rozengurt E.** Phorbol esters and vasopressin stimulate DNA synthesis by a common mechanism. *Nature* 287: 607–612, 1980.
14. **Fabregat I and Rozengurt E.** Vasoactive intestinal contractor, a novel peptide, shares a common receptor with endothelin-1 and stimulates Ca^{2+} mobilization and DNA synthesis in Swiss 3T3 cells. *Biochem Biophys Res Commun* 167: 161–167, 1990.
15. **Geli MI and Riezman H.** Endocytic internalization in yeast and animal cells: similar and different. *J Cell Sci* 111: 1031–1037, 1998.
16. **Geli MI and Riezman H.** Role of type I myosins in receptor-mediated endocytosis in yeast. *Science* 272: 533–535, 1996.
17. **Goldstein JL, Brown MS, Anderson RG, Russell DW, and Schneider WJ.** Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu Rev Cell Biol* 1: 1–39, 1985.
18. **Gottlieb TA, Ivanov IE, Adesnik M, and Sabatini DD.** Actin microfilaments play a critical role in endocytosis at the apical but not the basolateral surface of polarized epithelial cells. *J Cell Biol* 120: 695–710, 1993.
19. **Grady EF, Slice LW, Brant WO, Walsh JH, Payan DG, and Bunnett NW.** Direct observation of endocytosis of gastrin releasing peptide and its receptor. *J Biol Chem* 270: 4603–4611, 1995.
20. **Grynkiewicz G, Poenie M, and Tsien RY.** A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440–3450, 1985.
21. **Hall A.** Rho GTPases and the actin cytoskeleton. *Science* 279: 509–554, 1998.
22. **Jackman MR, Shurety W, Ellis JA, and Luzio JP.** Inhibition of apical but not basolateral endocytosis of ricin and folate in Caco-2 cells by cytochalasin D. *J Cell Sci* 107: 2547–2556, 1994.
23. **Kèubler E and Riezman H.** Actin and fimbrin are required for the internalization step of endocytosis in yeast. *EMBO J* 12: 2855–2862, 1993.
24. **Kroog GS, Jian X, Chen L, Northup JK, and Battey JF.** Phosphorylation uncouples the gastrin-releasing peptide receptor from G(q). *J Biol Chem* 274: 36700–36706, 1999.
25. **Kroog GS, Sainz E, Worland PJ, Akeson MA, Benya RV, Jensen RT, and Battey JF.** The gastrin-releasing peptide receptor is rapidly phosphorylated by a kinase other than protein kinase C after exposure to agonist. *J Biol Chem* 270: 8217–8124, 1995.
26. **Lamaze C, Fujimoto LM, Yin HL, and Schmid SL.** The actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells. *J Biol Chem* 272: 20332–20335, 1997.
27. **Lamaze C and Schmid SL.** Recruitment of epidermal growth factor receptors into coated pits requires their activated tyrosine kinase. *J Cell Biol* 129: 47–54, 1995.
28. **Lefkowitz RJ.** G Protein-coupled receptors. III. New roles for receptor kinases and B-arrestins in receptor signaling and desensitization. *J Biol Chem* 273: 18677–18680, 1998.
29. **Munn AL, Stevenson BJ, Geli MI, and Riezman H.** end5, end6, and end7: mutations that cause actin delocalization and block the internalization step of endocytosis in Saccharomyces cerevisiae. *Mol Biol Cell* 6: 1721–1742, 1995.
30. **Needham LK and Rozengurt E.** $G\alpha_{12}$ and $G\alpha_{13}$ stimulate Rho-dependent tyrosine phosphorylation of focal adhesion kinase, paxillin, and p130 Crk-associated substrate. *J Biol Chem* 273: 14626–14632, 1998.
31. **Patterson RL, van Rossum DB, and Gill DL.** Store-operated Ca^{2+} entry: evidence for a secretion-like coupling model. *Cell* 98: 487–499, 1999.
32. **Paul BZ, Daniel JL, and Kunapuli SP.** Platelet shape change is mediated by both calcium-dependent and -independent signaling pathways. Role of p160 Rho-associated coiled-coil-containing protein kinase in platelet shape change. *J Biol Chem* 274: 28293–28300, 1999.
33. **Ridley AJ and Hall A.** The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70: 389–399, 1992.

34. **Rodríguez-Fernández JL and Rozengurt E.** Bombesin, vasopressin, lysophosphatidic acid, and sphingosylphosphorylcholine induce focal adhesion kinase activation in intact Swiss 3T3 cells. *J Biol Chem* 273: 19321–19328, 1998.
35. **Rozengurt E.** Early signals in the mitogenic response. *Science* 234: 161–166, 1986.
36. **Rozengurt E.** Gastrointestinal peptide signaling through tyrosine phosphorylation of focal adhesion proteins. *Am J Physiol Gastrointest Liver Physiol* 275: G177–G182, 1998.
37. **Schmidt M, Voss M, Weernink PA, Wetzel J, Amano M, Kaibuchi K, and Jakobs KH.** A role for rho-kinase in rho-controlled phospholipase D stimulation by the m3 muscarinic acetylcholine receptor. *J Biol Chem* 274: 14648–14654, 1999.
38. **Sinnett-Smith J, Zachary I, Valverde AM, and Rozengurt E.** Bombesin stimulation of p125 focal adhesion kinase tyrosine phosphorylation. Role of protein kinase C, Ca^{2+} mobilization, and the actin cytoskeleton. *J Biol Chem* 268: 14261–14268, 1993.
39. **Sorkina T, Bild A, Tebar F, and Sorkin A.** Clathrin, adaptors and eps15 in endosomes containing activated epidermal growth factor receptors. *J Cell Sci* 112: 317–327, 1999.
40. **Spector I, Shochet NR, Blasberger D, and Kashman Y.** Latrunculins: novel marine macrolides that disrupt microfilament organization and affect cell growth. I. Comparison with cytochalasin D. *Cell Motil Cytoskeleton* 13: 127–144, 1989.
41. **Wells A, Welsh JB, Lazar CS, Wiley HS, Gill GN, and Rosenfeld MG.** Ligand-induced transformation by a noninternalizing epidermal growth factor receptor. *Science* 247: 962–964, 1990.
42. **Wiley HS, Herbst JJ, Walsh BJ, Lauffenburger DA, Rosenfeld MG, and Gill GN.** The role of tyrosine kinase activity in endocytosis, compartmentation, and down-regulation of the epidermal growth factor receptor. *J Biol Chem* 266: 11083–11094, 1991.
43. **Zachary I and Rozengurt E.** Identification of a receptor for peptides of the bombesin family in Swiss 3T3 cells by affinity cross-linking. *J Biol Chem* 262: 3947–3950, 1987.
44. **Zachary I and Rozengurt E.** Internalization and degradation of peptides of the bombesin family in Swiss 3T3 cells occurs without ligand-induced receptor down-regulation. *EMBO J* 6: 2233–2239, 1987.
45. **Zachary I, Sinnett-Smith J, and Rozengurt E.** Bombesin, vasopressin, and endothelin stimulation of tyrosine phosphorylation in Swiss 3T3 cells. Identification of a novel tyrosine kinase as a major substrate. *J Biol Chem* 267: 19031–19034, 1992.
46. **Zachary I, Sinnett-Smith J, Turner CE, and Rozengurt E.** Bombesin, vasopressin, and endothelin rapidly stimulate tyrosine phosphorylation of the focal adhesion-associated protein paxillin in Swiss 3T3 cells. *J Biol Chem* 268: 22060–22065, 1993.