β1-Adrenergic receptor (β1AR) stimulation confers cardioprotection via β-arrestin-dependent transactivation of epidermal growth factor receptors (EGFRs), however, the precise mechanism for this salutary process is unknown. We tested the hypothesis that the β1AR and EGFR form a complex that differentially directs intracellular signaling pathways. β1AR stimulation and EGF ligand can each induce equivalent EGFR phosphorylation, internalization, and downstream activation of ERK1/2, but only EGF ligand causes translocation of activated ERK to the nucleus, whereas β1AR-stimulated/EGFR-transactivated ERK is restricted to the cytoplasm. β1AR and EGFR are shown to interact as a receptor complex both in cell culture and endogenously in human heart, an interaction that is selective and undergoes dynamic regulation by ligand stimulation. Although catecholamine stimulation mediates the retention of β1AR-EGFR interaction throughout receptor internalization, direct EGF ligand stimulation initiates the internalization of EGFR alone. Continued interaction of β1AR with EGFR following activation is dependent upon C-terminal tail GRK phosphorylation sites of the β1AR and recruitment of β-arrestin. These data reveal a new signaling paradigm in which β-arrestin is required for the maintenance of a β1AR-EGFR interaction that can direct cytosolic targeting of ERK in response to catecholamine stimulation.

β1-Adrenergic receptor (β1AR) stimulation regulates a number of signaling pathways and has been recently shown to transactivate epidermal growth factor receptor (EGFR) and increase ERK activation in both in vitro cell culture systems and in vivo mouse models (1). The key signaling components required for EGFR transactivation following β1AR stimulation include: 1) C-terminal phosphorylation of activated β1ARs by GRK5/6, and 2) recruitment of both β-arrestins 1 and 2 to phosphorylated β1ARs. Recruitment of β-arrestins to activated β1ARs allow subsequent activation of c-Src and matrix metalloproteases, cleavage of HB-EGF and activation of EGFR, processes that contribute to transactivation pathways defined for other 7TMRs (2–7). Trafficking of EGFR has been shown to be critical in defining downstream signaling pathways regulated by ligand-induced activation (8). Although direct EGFR stimulation via its ligand EGF leads to internalization of the receptor as well as ERK1/2 translocation to the nucleus and activation of Elk-1-mediated transcription (9, 10), the precise mechanism by which ERK signaling pathways are regulated following β1AR-mediated EGFR stimulation is poorly understood.

Recruitment of β-arrestin1/2 to activated 7TMRs allows these multifunctional scaffold proteins to target other signaling proteins that are involved in receptor internalization, desensitization, and intracellular signaling complexes. Members of the mitogen-activated protein kinase family such as ERK are among the proteins recruited to receptors by β-arrestins (11). Several recent studies have shown that β-arrestin1/2 recruitment to activated 7TMRs may direct both cytosolic ERK1/2 signaling, via formation of internalized β-arrestin signalosomes containing the receptor and activated ERK (12, 13), and nuclear targeting of ERK (14, 15), depending upon the 7TMR, cell-type, and culture conditions tested. Although β-arrestin recruitment is essential for β1AR-mediated transactivation of EGFR, it remains unclear if β-arrestins play a role in regulating and targeting the downstream ERK response.

In this study we sought to determine whether the mode of EGFR activation, via β1AR-mediated transactivation versus direct ligand activation, induces differential effects on ERK1/2 signaling and if so, to elucidate the mechanism by which this process occurs. Here, we show that β1AR and EGFR form a receptor complex at the plasma membrane that is dynamically regulated by ligand stimulation, leading to differential ERK1/2 targeting and intracellular effects. Moreover, the recruitment
of β-arrestin to the agonist-occupied β1AR is required to maintain prolonged β1AR-EGFR interaction during simultaneous receptor internalization and to retain ERK1/2 activation in the cytosol. These findings illustrate the importance of β-arrestin in mediating receptor-receptor interaction and the targeting of downstream signaling pathways.

EXPERIMENTAL PROCEDURES

**Plasmids**—WT-β1AR and GRK−β1AR plasmids have been described (16). FLAG-EGFR, EGFR-GFP, ERK2-RFP, Myr-Palm-mYFP, and βarr2-mYFP plasmids and HA-AT1AR cells were provided by Dr. Robert J. Lefkowitz, Duke University. βarr2-RRK/Q-GFP was a gift of Dr. Christopher D. Nelson, Duke University. Mouse β1AR and human EGFR were ligated into mCFP or mYFP vectors with Xhol and HindIII using T4 ligase (Promega). pFA2-ELK1 and pFA2-CREB vectors were purchased from Stratagene, and pGL4-firefly luciferase and pRL-TK Renilla luciferase from Promega.

**Cell Culture**—WT-β1AR, GRK−β1AR, and β1AR-mCFF cells (each ~1 pmol/mg protein), HA-AT1AR cells (~2 pmol/mg protein), or native cells were grown in minimal essential medium, 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C and transfected using FuGENE 6 (Roche Diagnostics). Cells were incubated overnight in serum-free medium and pretreated with ICI-118,551 (ICI, FL Arrestin2 antagonist, 0.1 μM, 5 min, Sigma) ± AG 1478 (EGFR inhibitor, 1 μM, 5 min, Calbiochem) or H89 (PKA inhibitor, 1 μM, 5 min, Sigma) followed by agonists.

**Confocal Laser Microscopy**—After treatment, cells were phosphate-buffered saline-rinsed, fixed in 4% paraformaldehyde for 20 min, permeabilized with ice-cold 0.2% Triton X-100 for 5 min, rinsed, and blocked with 1% bovine serum albumin for 1 h. Incubation with anti-phosphorylated ERK1/2 rabbit antibody (1:250 for 16 h, Cell Signaling) or anti-FLAG mouse antibody (1:10000 for 20 min, Sigma) ± AG 1478 (EGFR inhibitor, 1 μM, 5 min, Calbiochem) or H89 (PKA inhibitor, 1 μM, 5 min, Sigma) followed by agonists.

**Fluorescence Resonance Energy Transfer (FRET)**—Imaging buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgCl2, 1.5 mM CaCl2, 10 mM glucose, 0.2% bovine serum albumin, 10 mM HEPES, pH 7.4) was added to β1AR-mCFF cells expressing EGFR-mYFP in 35-mm dishes on a 37 °C heated stage. Images were acquired on a Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc.) with a Roper Micromax cooled charge-coupled device camera (Photometrics) and SlideBook 4.0 (Intelligent Imaging Innovations). Agonist-induced increases or decreases in FRET was monitored using cells emitting ~10% basal FRET. All calculations used have been described (22).

**β-Arrestin1/2 siRNA Experiments**—Control (CTL) and β-arr1 and β-arr2 siRNA sequences and protocol have been used by us previously and described (1, 17, 23). For microscopy experiments, 35-mm dishes of WT-β1AR cells or β1AR-mCFF cells were transfected with CTL or a combination of β-arr1 and β-arr2 siRNA (7 μg) and EGFR-GFP or EGFR-mYFP (0.3 μg). After FRET, cells were lysed and immunoblotted to confirm β-arr1/2 knockdown.

**Statistical Analysis**—Statistical tests were performed using either two-tailed unpaired t test or one-way analysis of variance with the Newman-Keuls multiple comparison post hoc test using Prism 5.0 software. p value (*, p < 0.05; †, p < 0.01, ‡, p < 0.001) of <0.05 was considered significant.

RESULTS

**β1AR-mediated Transactivation of EGFR Induces Cytosolic Retention of ERK**—We tested whether the mechanism of EGFR activation, β1AR-mediated transactivation by catecholamine (Dob or ISO) versus direct ligand stimulation (EGF), differentially effects intracellular ERK targeting. HEK 293 cells stably expressing FLAG-tagged WT-β1AR (WT-β1AR cells) and
transiently expressing green fluorescent protein-tagged EGFR (EGFR-GFP) and red fluorescent protein-tagged ERK2 (ERK2-RFP) were stimulated with agonists and assessed via confocal microscopy. In a non-stimulated state, EGFR-GFP was localized to the cell membrane, whereas ERK2-RFP was cytosolic (Fig. 1A, panels 1 and 2). Simulation for 20 min with Dob (β1AR agonist) or EGF (direct EGFR ligand) each induced internalization of EGFR-GFP as indicated by puncta formation (Fig. 1A, panels 3 and 5, arrowheads), which was prevented by pretreatment with AG 1478 (1 μM) (Fig. 1A, panels 7 and 9). Conversely, ERK2-RFP underwent differential targeting in response to agonist stimulation; EGF caused nuclear translocation of ERK2-RFP (Fig. 1A, panel 6, arrow), which was sensitive to AG 1478 (Fig. 1A, panel 10), whereas ERK remained in the cytosol following Dob stimulation (Fig. 1A, panel 4). AG 1478 did not alter ERK2-RFP targeting in response to Dob (Fig. 1A, panel 8). H89 had no effect on agonist-mediated EGFR-GFP internalization (Fig. 1A, panels 11 and 13, arrowheads) or ERK2-RFP targeting (Fig. 1A, panels 12 and 14, arrow), indicating that these effects are independent of PKA activity downstream of G protein signaling. In addition, we tested whether stimulation of β1ARs that lack GRK phosphorylation sites in the C-terminal tail (GRK−β1AR) induce a different outcome on ERK translocation. We have previously shown GRK−β1ARs are significantly impaired in their ability to induce EGFR transactivation and ERK1/2 phosphorylation (1, 17). Consistent with these previous observations, Dob stimulation of GRK−β1AR cells did not induce EGFR-GFP internalization (panel 15) nor altered ERK2-RFP localization (panel 16), whereas EGF stimulation did induce both EGFR-GFP internalization (panel 17, arrowheads) and nuclear translocation of ERK2-RFP (panel 18, arrows). Scale bar = 10 μm. Images shown are representative of at least four independent experiments. B, Dob (1 μM) or EGF (1 nm) stimulation for 5 min increased P-ERK1/2 in WT-β1AR cells transfected with FLAG-EGFR, whereas AG 1478 (1 μM) pretreatment attenuated P-ERK1/2 as shown in the histogram. Data represent the mean ± S.E. from at least five independent experiments. ‡, p < 0.001. C, Dob (1 μM) stimulation for 5 min increased P-ERK1/2, which was not significantly attenuated by H89 (1 μM), whereas pretreatment with H89 and AG 1478 (1 μM) significantly attenuated phosphorylation as summarized in the histogram. Data represent the mean ± S.E. from four independent experiments. ‡, p < 0.001. D, WT-β1AR cells stimulated with Dob (1 μM) or EGF (1 nm) for 5 min underwent cellular fractionation (confirmed by FLAG-β1AR and Lamin A/C enrichment). Dob induced cytosolic P-ERK1/2 only, whereas EGF increased both cytosolic and nuclear P-ERK1/2, summarized in the histogram. Data represent the mean ± S.E. from six independent experiments. *, p < 0.05; ‡, p < 0.001. E, WT-β1AR cells transiently expressing FLAG-EGFR were stimulated with ISO (1 μM) or EGF (1 nm) for 5 min in the presence or absence of AG 1478 (1 μM) and phospho (P)-p75/85 S6K was measured via immunoblotting. Results are representative of three independent experiments. IB, immunoblot.
β1AR-EGFR Interaction Regulates ERK Trafficking

Dob-induced P-ERK1/2 levels were reduced by ~25% indicating that PKA-dependent signaling is responsible for only a small portion of P-ERK1/2 with the majority accounted for by β1AR-mediated EGFR transactivation. Additionally, cell fractionation experiments confirmed that Dob significantly induced only cytosolic ERK1/2 phosphorylation, whereas EGF significantly increased both cytosolic and nuclear ERK1/2 phosphorylation (Fig. 1D). Indeed, p75/p85 S6 kinase (S6K), a downstream target of cytosolic P-ERK, is phosphorylated in response to both catecholamine and EGF stimulation in an AG1478-sensitive (sp) manner (Fig. 1E).

Consistent with the above data, Dob stimulation resulted in P-ERK2-RFP being targeted only to the cytosol (Fig. 2A, panel 3), whereas EGF stimulation resulted in the targeting of P-ERK2-RFP to both cytosol and nucleus (Fig. 2A, panel 5, arrow). Each of these responses was abrogated with the inclusion of AG1478 (Fig. 2A, panels 7 and 9). We then tested whether nuclear translocation of ERK1/2 would induce gene transcription as measured by Elk-1-Gal4 luciferase reporter activity. EGF stimulation resulted in a significant increase in the amount of Elk-1-Gal4-driven luciferase activity, indicative of increased ERK1/2 activity in the nucleus (Fig. 2B), which was blocked by AG1478 pretreatment. Conversely, activation of Elk-1-Gal4 luciferase was not observed with Dob (Fig. 2B) or ISO (Fig. 2D). To ensure catecholamine stimulation produced a β1AR-mediated signal in these assays, CREB-Gal4 luciferase activity was assessed, indicative of cAMP signaling downstream of β1AR activation. Using this system, both Dob (Fig. 2C) and ISO (Fig. 2D) caused significant induction of CREB-Gal4-mediated luciferase activity. Therefore, whereas β1AR-mediated transactivation and direct EGF ligand stimulation each induce EGFR internalization and downstream phosphorylation of ERK, these stimuli result in differential intracellular targeting and function of ERK1/2.

**Ligand Concentrations Matched for EGFR and ERK1/2 Activation Maintain Differential ERK Targeting**—HB-EGF is an endogenously expressed membrane protein known to be cleaved by matrix metalloproteinases in response to βAR stimulation (1, 4, 17). To test the effect of bypassing β1AR-mediated EGFR transactivation with exogenously added HB-EGF and EGF, we measured ERK1/2 activation and cellular targeting at various time points following HB-EGF, EGF, and Dob stimulation. In WT-β1AR cells transiently transfected with FLAG-
0.01 nM HB-EGF resulted in robust nuclear accumulation of ERK2-RFP (Fig. 4B). To assess the effects of increasing concentrations of Dob, EGF, and HB-EGF on EGFR-GFP internalization, we calculated the loss of EGFR-GFP from the plasma membrane in response to ligand stimulation (Fig. 4C). The concentrations of 0.1 nM EGF and 0.01 nM HB-EGF induced equivalent EGFR-GFP internalization as Dob at concentrations ≥1 μM. Consistent with the internalization data, the level of P-EGFR in response to 1 μM Dob was equivalent to that of 0.1 nM EGF indicating similar EGFR activation (Fig. 4D). To determine whether the concentrations of exogenous HB-EGF added in these experiments were within the range of endogenous HB-EGF shed in response to β1AR activation, we collected the media of WT-β1AR cells stimulated with Dob for 5 min and measured the amount of HB-EGF released (Fig. 4E). Under non-stimulated conditions, cells released a basal level of 0.94 ± 0.15 pM HB-EGF into the media. In contrast, the media of Dob-stimulated cells contained 3.54 ± 0.13 pM HB-EGF. Thus, whereas Dob causes release of HB-EGF into the media that induces a similar level of EGFR internalization and P-ERK1/2 as 0.01 nM HB-EGF, differential mechanisms must be involved to account for the subsequent cytosolic retention of ERK.

β1AR and EGFR Associate with Specificity and Their Association Is Differentially Regulated by Ligand Stimulation—To address the mechanism by which differential targeting of ERK in response to ligand may occur, we tested the possibility that β1AR and EGFR may interact and direct the cellular localization of ERK. Recently, a number of studies have reported an interaction between other 7TMRs and EGFR (5, 24–28). In FLAG-β1AR cells transfected with EGFR-GFP, IP of FLAG-β1AR resulted in co-IP of EGFR (Fig. 5A). In contrast, EGFR-GFP overexpression in HEK 293 cells stably expressing the hemagglutinin-tagged angiotensin type 1A receptor (HA-AT1AR) did not result in the co-IP of EGFR with AT1AR, even following angiotensin II stimulation (Fig. 5B). Angiotensin II stimulation did induce association between AT1AR and ERK2-RFP, confirming receptor activation and ability to interact with predicted proteins. Thus, simple overexpression of another 7TMR (AT1AR), which is known to transactivate EGFR (27, 29, 30), is insufficient to induce its interaction with EGFR in this system.

FIGURE 3. Time-dependent activation of ERK1/2 by β1AR-mediated transactivation and direct ligand stimulation of EGFR. A, WT-β1AR cells transfected with FLAG-EGFR were stimulated with Dob (10 μM), HB-EGF (1 nM), or EGF (1 nM) for 0 – 60 min and cell lysates immunoblotted (IB) for P-ERK1/2 and T-ERK1/2. The histogram depicts the time course of P-ERK1/2 response for each ligand. Data represent the mean ± S.E. from at least four independent experiments. *, p < 0.05; †, p < 0.01; ‡, p < 0.001. B, WT-β1AR cells were transiently transfected with EGFR-GFP and ERK2-RFP and stimulated with Dob (1 μM), HB-EGF (1 nM), or EGF (1 nM) for 0 – 60 min. Dob (panels 3 and 4), HB-EGF (panels 6–8), and EGF (panels 10–12) each induced EGFR-GFP internalization (arrowheads), whereas only HB-EGF (panels 6–8) and EGF (panels 10–12) caused translocation of ERK2-RFP to the nucleus (arrows). Scale bars = 10 μm. Images shown are representative of at least four independent experiments. C, WT-β1AR cells were transiently transfected with ERK2-RFP and stained with anti-P-ERK1/2 antibody following Dob (1 μM) or EGF (1 nM) stimulation for increasing periods of time. Peak ERK2-RFP phosphorylation was achieved within 5 min for both Dob (panel 4) and EGF (panel 6). EGF stimulation induced prolonged P-ERK2-RFP in both the cytosol and the nucleus. Scale bar = 10 μm. Composite images shown are representative of at least four independent experiments.
**β1AR-EGFR Interaction Regulates ERK Trafficking**

To determine whether β1AR and EGFR interact at endogenous levels and *in vivo*, we performed co-IP experiments in U2S cells and human heart tissue, which basally express both the EGFR and ~35–60 fmol/mg protein of βARs (Fig. 5C). Membrane preparations from both U2S cells and heart tissue were used to IP endogenous EGFR followed by radioligand binding with the highly specific βAR ligand [125I]iodocyanopindolol. Nonspecific binding was determined by the separate addition of IgG followed by IP. Within the EGFR immunoprecipitates we detected a significant level of endogenous βAR compared with IgG control immunoprecipitates in both U2S cells (0.16 ± 0.04 pmol of receptor, Fig. 5D) and human heart tissue (0.13 ± 0.06 pmol of receptor, Fig. 5E). These experiments support our data that under basal conditions there is an interaction between β1ARs and EGFRs.

To determine the specificity of β1AR-EGFR interaction, we performed FRET experiments in HEK 293 cells stably expressing monomeric cyan fluorescent protein-tagged β1AR (β1AR-mCFP) cells and transiently expressing either EGFR-mYFP or myristoylated-palmitylated mYFP (MyrPalm-mYFP). MyrPalm-mYFP is targeted to the plasma membrane (31), thereby providing a nonspecific membrane-bound FRET partner for β1AR-mCFP for comparison with EGFR-mYFP to determine specificity of interaction. As levels of MyrPalm-mYFP and EGFR-mYFP increased, the amount of detectable FRET also increased, achieving maximal %FRET of ~50 and 20%, respectively (Fig. 5F). Because the maximal FRET efficiency between β1AR-mCFP and either mYFP-tagged protein is influenced by both proximity and orientation of mCFP and mYFP, and dependent upon mYFP concentration, it does not necessarily represent the true amount of specific interaction between the partners. Therefore, we compared the relative affinities of EGFR-mYFP and MyrPalm-mYFP for β1AR-mCFP calculated via saturation binding analysis. A 5-fold greater affinity of EGFR for β1AR than that of MyrPalm-mYFP for β1AR-mCFP was attained (Fig. 5G), indicating that the interaction of β1AR with EGFR has significantly higher specificity than with a general membrane-bound protein.

We next used FRET analysis to explore the possibility that agonist stimulation can regulate β1AR-mCFP-EGFR-mYFP association. Catecholamine stimulation (either Dob or ISO)
induced a small reduction in FRET over time that was indistinguishable from non-stimulated cells (Fig. 5H). In contrast, EGF stimulation caused an immediate decrease in FRET signal that persisted. Nonlinear regression analysis of the data revealed approximately a 5-fold greater loss in FRET (\%F_{\text{max}}) with EGF stimulation compared with Dob or ISO (Fig. 5I). Thus, EGF stimulation significantly disrupts \( \beta 1 \text{AR-EGFR} \) interaction, whereas catecholamine stimulation maintains receptor interaction.

**\( \beta 1 \text{AR and EGF} \) Interaction Is Regulated by C-terminal GRK Phosphorylation Sites and \( \beta \)-Arrin-mediated Internalization—GRK-mediated phosphorylation of the \( \beta 1 \text{AR} \) is required for transactivation of EGF by inducing \( \beta \)-arrestin recruitment (1). We have previously shown that GRK-\( \beta 1 \text{ARs} \) are significantly impaired in their ability to induce EGF transactivation and ERK1/2 phosphorylation (1, 17). Therefore, we tested whether \( \beta 1 \text{AR} \) GRK phosphorylation sites are required for \( \beta 1 \text{AR-EGFR} \) interaction. In non-stimulated WT-\( \beta 1 \text{AR} \) cells transfected with EGF-GFP, \( \beta 1 \text{AR} \) and EGF were expressed on the plasma membrane (Fig. 6A, panels 1 and 2), and co-localize in puncta upon stimulation with ISO (Fig. 6A, panels 3 and 4, arrowheads). In contrast, EGF stimulation caused EGF-GFP internalization alone, whereas \( \beta 1 \text{ARs} \) remained on the cell surface (Fig. 6A, panels 5 and 6, arrowheads). Because GRK-\( \beta 1 \text{ARs} \) can undergo catecholamine-stimulated internalization by a clathrin/\( \beta \)-arrestin-independent mechanism (16), we tested whether they would co-localize with EGF. ISO stimulation induced internalization of GRK-\( \beta 1 \text{AR}, \) leaving EGF-GFP at the cell surface (Fig. 6A, panels 9 and 10, arrowheads), whereas EGF stimulation again produced internalization of EGF-GFP without inducing GRK-\( \beta 1 \text{AR} \) internalization (Fig. 6A, panels 11 and 12, arrowheads). Thus, GRK phosphorylation sites in the \( \beta 1 \text{AR} \) are essential for \( \beta 1 \text{AR}-
EGFR interaction following catecholamine stimulation. Additionally, we assessed the requirement of β1AR GRK phosphorylation sites for the basal association of β1AR and EGFR. HEK 293 cells stably expressing either FLAG-tagged WT-β1AR or GRK−β1AR and transfected with EGFR-GFP underwent IP with FLAG M2-agarose and the amount of EGFR-GFP associated with each β1AR was assessed. In comparison to WT-β1AR, GRK−β1AR had a significantly decreased ability to associate with EGFR-GFP (Fig. 6B). Densitometric analysis revealed a decrease of ~90% in β1AR-EGFR association when
β1AR GRK phosphorylation sites were absent, suggesting the C-terminal conformation of β1AR may be an important factor in determining its interaction with EGFR.

To explore the possible requirement of β-arrestin in mediating β1AR-EGFR trafficking after catecholamine stimulation, we assessed the ability of WT-β1AR and GRK-β1AR cells to recruit β-arrestin. Stimulation with catecholamine induced a significant rapid increase in β-arrestin recruitment to the WT-β1AR, peaking at 5 min and remaining elevated up to 30 min post-stimulation (Fig. 6C). Importantly, stimulation with EGF in WT-β1AR cells or with ISO in GRK-β1AR cells did not result in β-arrestin association suggesting that β-arrestin recruitment to the receptor complex is required for their continued association in the presence of catecholamine.

We next tested the possibility that β1AR-EGFR co-localization following catecholamine stimulation depends on β-arrestin-mediated internalization of the receptor complex. We used a β-arrestin2 mutant with altered C-terminal amino acid residues (RRK/Q) that render it unable to induce receptor internalization despite retaining its ability to be recruited to activated βAR (32, 33). To ensure this β-arrestin2 mutant could be recruited to agonist-stimulated β1ARs in our system, we treated WT-β1AR cells transfected with either WT-βarr2-mYFP or RRK/Q-β-arrestin2-GFP with ISO and assessed β-arrestin2 recruitment via confocal microscopy (Fig. 6D). ISO stimulation induced the recruitment of both WT- and RRK/Q-β-arrestin2 to β1ARs. Subsequently, performing FLAG-β1AR IP, we compared the effects of WT- and RRK/Q-β-arrestin2 on β1AR-EGFR association in WT-β1AR cells transfected with EGFR-GFP and either WT-β-arrestin2-mYFP or RRK/Q-β-arrestin2-GFP (Fig. 6E). In non-stimulated cells EGFR associated with β1AR in the presence of either WT- or RRK/ Q-β-arrestin2. Following β1AR stimulation, recruitment of both WT- and RRK/Q-β-arrestin2 increased similarly. Association of β1AR and EGFR did not change in the presence of WT-βarr2, however, in the presence of RRK/Q-β-arrestin2, ISO stimulation caused a significant decrease in β1AR-EGFR interaction (78.6 ± 6.4%)

** Knockdown of β-Arrestin1/2 Prevents β1AR-EGFR Interaction following Catecholamine Stimulation—Because overexpression of the internalization-deficient β-arrestin2 RRK/Q led to impairment of catecholamine-induced β1AR-EGFR association, we sought to determine whether knockdown of endogenous β-arrestin1/2 would perturb the trafficking of the β1AR-EGFR complex following catecholamine stimulation. FRET analysis was performed in β1AR-mCFP cells transfected with EGFR-mYFP and CTL siRNA or a combination of two siRNAs targeting either β-arrestin1 or β-arrestin2 (β-arrestin1/2 siRNA). Dob stimulation of cells that received CTL siRNA produced minimal loss of FRET over time (Fig. 7A), comparable with the response to Dob shown in Fig. 5H. In contrast, Dob stimulation of cells transfected with β-arrestin1/2 siRNA, causing ≥90% ablation of β-arrestin1/2 expression, reduced calculated %F max by 2.55 ± 0.11%, as determined by nonlinear regression analysis.

Furthermore, we investigated the co-localization of β1AR and EGFR in the presence of CTL versus β-arrestin1/2 siRNA via confocal microscopy. Cells transfected with either siRNA maintained β1AR and EGFR co-localization at the cell surface in a non-stimulated state (Fig. 7B, panels 1, 2, 5, and 6). Treatment with ISO caused the formation of internalized puncta containing both β1AR and EGFR in CTL siRNA-treated cells (Fig. 7B, panels 3 and 4, arrowheads), but only β1AR in β-arrestin1/2 siRNA-treated cells (Fig. 7B, panels 7 and 8, arrowhead). EGF stimulation induced internalization of only EGFR-GFP in both CTL and β-arrestin1/2 siRNA-treated cells (not shown). These data demonstrate that whereas the basal association of receptors at the cell surface does not require β-arrestin1/2, following catecholamine stimulation endogenous β-arrestin1/2 recruitment is necessary to maintain β1AR-EGFR interaction and co-internalization.

To determine whether β-arrestin1/2 interaction with the β1AR-EGFR complex directs ERK signaling, IP of FLAG-β1AR with or without catecholamine stimulation was performed in the presence of CTL or β-arrestin1/2 siRNA and associated proteins assessed. In WT-β1AR cells transfected with EGFR-GFP and CTL siRNA, EGFR associated with β1AR before and after ISO stimulation (Fig. 7C), corroborating our FRET and confocal microscopy data. β-arrestin1/2 and P-ERK1/2 were observed to interact with β1AR only following catecholamine stimulation. In accordance with our previous studies (1, 17), β-arrestin1/2 siRNA abolished the P-ERK1/2 response to catecholamine stimulation in the cell lysate. Overall, β-arrestin1/2 siRNA mediated the loss of β1AR association with EGFR.
**Discussion**

Although 7TMR-mediated transactivation of EGFR has been shown to induce phosphorylation of ERK1/2, the mechanisms responsible for this effect vary as widely as the 7TMRs that transactivate EGFR (17, 24, 28, 34, 35). Previously, we have shown a role for β-arrestin in the regulation of β1AR-mediated transactivation of EGFR (1, 17) and in this study we show that β1AR and EGFR interact as a complex whose continued association following agonist stimulation is dependent upon GRK phosphorylation sites in the C-terminal tail of β1AR, and on the recruitment of β-arrestin. We identify that β1AR-mediated EGFR transactivation leads to differential intracellular trafficking of ERK1/2 compared with direct ligand stimulation of EGFR. Each stimulus induces EGFR activation and internalization, but despite this common feature, the biological consequences of these distinct stimuli are divergent. Others have reported that β-arrestin functions as a scaffold for ERK and their upstream kinases, thus providing a pool of β-arrestin-bound ERK1/2 in the cytosol (36–38). Thus, we propose that upon cate-

![Image](https://example.com/image.png)
cholamine stimulation, GRK-mediated phosphorylation of the cytoplasmic tail of β1AR favors recruitment of βar1/2-bound ERK1/2 over free cytosolic ERK1/2. β-Arrestin induces transactivation of EGFR, which allows for phosphorylation of the β-arrestin-recruited ERK1/2. Following β-arrestin-mediated internalization of the β1AR-EGFR complex, β-arrestin dissociates from the receptor complex and restricts bound P-ERK1/2 to the cytosol (Fig. 7D). Whether ERK1/2 phosphorylation is dependent on β1AR-EGFR internalization, or occurs simultaneously but independent of receptor internalization, is not known. Direct ligand stimulation of EGFR with EGF, however, does not initiate GRK or β-arrestin signaling, but induces dissociation of EGFR from β1AR, recruitment and phosphorylation of non-β-ar1/2-bound ERK1/2, and internalization of EGFR away from the plasma membrane. This pool of P-ERK1/2 is targeted throughout both the cytosol and nucleus (Fig. 7E). These findings provide new insight into the mechanism of β-arrestin-mediated signaling following catecholamine stimulation, which only induces EGFR transactivation, but also induces internalization of a β1AR-EGFR complex and directs intracellular ERK1/2 targeting.

The dynamics of ERK1/2 phosphorylation in response to 7TMR stimulation, including AT1R, β2AR, and parathyroid hormone receptor, have been studied in detail by others (12, 39, 40). Both β-arrestin-dependent and -independent signaling pathways contribute to phosphorylation of ERK1/2, however, the extent to which each pathway induces this effect differs for various 7TMRs. In our study we show that, in response to β1AR stimulation, a majority of the resulting ERK1/2 phosphorylation is dependent on β-arrestin-mediated EGFR transactivation. Approximately 70–75% of catecholamine-induced P-ERK1/2 was abolished via inhibition of EGFR with AG 1478 (Fig. 1B) or siRNA-directed ablation of β-arrestin1/2 expression (Fig. 7C). Consistent with these data, blocking PKA signaling with H89 reduced the P-ERK1/2 response by ~25% following β1AR stimulation (Fig. 1C). In terms of β1AR-mediated EGFR transactivation, inhibition of PKA signaling with H89 had no effect on EGFR internalization, whereas blockade of EGFR with AG 1478 or activation of GRK-β1AR (Fig. 1A) or knockdown of β-ar1/2 completely prevented this process (Fig. 7B). Thus, whereas β-arrestin-independent signaling plays a small role in ERK1/2 phosphorylation following β1AR stimulation, the majority of ERK1/2 activation is achieved via β-arrestin-dependent EGFR transactivation.

EGFR is known to undergo rapid internalization and targeting for recycling or degradation following stimulation with EGF in a concentration-dependent manner (8, 41, 42). Catecholamine stimulation of βARs induces a β-arrestin-associated internalization that leads to receptor recycling (11). Our data demonstrate co-localization of β1AR, EGFR, and β-arrestin following adrenergic stimulation that maintains cytosolic ERK1/2 signaling. Interestingly, even low concentrations of EGF or HB-EGF, which produced similar P-ERK1/2 levels as β1AR stimulation, did not lead to cytosolic-restricted ERK1/2. Therefore, β-arrestin recruitment to the receptor complex is necessary to induce EGFR transactivation, as we previously reported (1, 17), and to subsequently promote the maintenance of the receptor complex and cytosolic retention of ERK1/2. Indeed, our data demonstrate that an internalization-deficient β-arrestin mutant (RRK/Q), or the absence of β-arrestins altogether (siRNA knockdown), decreases β1AR-EGFR association by ~80% following catecholamine stimulation and significantly reduces interaction with phosphorylated ERK1/2 by ~70%. Thus, β-arrestin recruitment is essential not only for the induction of β1AR-mediated EGFR transactivation but also for directing β1AR-EGFR internalization and P-ERK1/2 trafficking in the cell. Conversely, direct ligand stimulation of EGFR does not recruit β-arrestins, leading to rapid dissociation of the receptor complex, internalization of EGFR alone, and simultaneous cytosolic and nuclear targeting of P-ERK1/2. Proximal to β-arrestin signaling, we also show a role for GRK phosphorylation sites in the regulation of β1AR-EGFR interaction, suggesting the C-terminal conformation of β1AR is important in allowing its interaction with EGFR.

ERK1 and ~2 lack both NLS and NES motifs, but do typically traffic into the nucleus following mitogenic stimulation via regulatory proteins (43). Indeed, several studies have shown that β-arrestins act as scaffolds for the components of ERK1/2 signaling following stimulation of various 7TMRs, including the AT1R, R and β2AR, and that stronger receptor-β-arrestin interactions usually lead to cytosolic retention of ERK1/2 (11, 12). In this study we show the cytosolic retention of ERK1/2 in response to β1AR stimulation is β-arrestin-dependent despite βARs classically having a lower affinity for β-arrestin (11). This may be reflective of either a small pool of β-arrestin-bound ERK1/2 available for phosphorylation via β1AR-mediated EGFR transactivation or the potential association of a protein phosphatase in the β1AR-β-arrestin1/2-EGFR-ERK1/2 complex, because β-arrestins have been shown to interact with several phosphatases (44).

The spectrum of 7TMRs that induce EGFR transactivation is an ever-expanding cohort that highlights the importance of EGFR as an alternative 7TMR signaling pathway beyond the classical G protein-dependent paradigm (1–7, 24–30, 35, 45–50). Although a number of 7TMRs have been shown to induce transactivation of EGFR, only a handful have been demonstrated to interact with EGFR, although the mechanism(s) of these associations have not been elucidated (5, 24–28). In our study we demonstrate a ligand-dependent, dynamic interaction between β1AR and EGFR using overexpression systems and show that this interaction occurs at endogenous receptor levels in cells and human heart tissue. Moreover, the recruitment of endogenous β-ar1/2 directs internalization of the β1AR-EGFR complex and is essential for their continued association upon catecholamine stimulation, because direct EGFR stimulation does not recruit β-arrestin and leads to the disruption of the receptor complex. Thus, our study provides new mechanistic insights into the regulation of this receptor complex by β-arrestin and the impact of this regulation on intracellular ERK1/2 targeting and activity. We propose that this dynamic β-arrestin-regulated receptor-receptor interaction may be a mechanism by which the β1AR can regulate EGFR signaling to exert differential cellular effects. Furthermore, we believe our findings may provide an attractive explanation for the cardiopro-
tective effects of β1AR-mediated EGFR transactivation we have previously observed (1).

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