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Acute Activation of β_2-Adrenergic Receptor Regulates Focal Adhesions through βArrestin2- and p115RhoGEF Protein-mediated Activation of RhoA*

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Background: β_2-Adrenergic receptors have been shown to regulate cancer cell migration, but the underlying mechanisms are not clear.
Results: Acute activation of β_2-adrenergic receptors elicits βArrestin2-dependent activation of p115RhoGEF, leading to RhoA activation and focal adhesion remodeling.
Conclusion: βArrestin2 plays important roles in the regulation of p115RhoGEF and activation of RhoA.
Significance: βArrestin2 may serve as a convergence point for non-$G_{12/13}$- or non-G_q-coupled receptors to regulate RhoA activity through p115RhoGEF.

β_2-Adrenergic receptors (β_2ARs) regulate cellular functions through G protein-transduced and βArrestin-transduced signals. β_2ARs have been shown to regulate cancer cell migration, but the underlying mechanisms are not well understood. Here, we report that β_2AR regulates formation of focal adhesions, whose dynamic remodeling is critical for directed cell migration. β_2ARs induce activation of RhoA, which is dependent on βArrestin2 but not G_s. βArrestin2 forms a complex with p115RhoGEF, a guanine nucleotide exchange factor for RhoA that is well known to be activated by $G_{12/13}$-coupled receptors. Our results show that βArrestin2 forms a complex with p115RhoGEF in the cytosol in resting cells. Upon β_2AR activation, both βArrestin2 and p115RhoGEF translocate to the plasma membrane, with concomitant activation of RhoA and formation of focal adhesions and stress fibers. Activation of RhoA and focal adhesion remodeling may explain, at least in part, the role of β_2ARs in cell migration. These results suggest that βArrestin2 may serve as a convergence point for non-$G_{12/13}$ and non-G_q protein-coupled receptors to activate RhoA.

Cancer cell metastasis involves multistep cellular processes, commencing with cell migration and invasion (1, 2). Cell migration is tightly regulated by coordinated remodeling of membrane and actin cytoskeleton. Focal adhesions are macromolecular structures that link actin cytoskeleton to the extracellular matrix and transmit force or tension. In addition, focal adhesions contain many associated signaling proteins that are involved in the regulation of cell proliferation, survival, and gene expression (3). Focal adhesions are constantly remodeled during cell migration. Polarized cells establish new adhesions at the front edge and release preexisting focal adhesions at the trailing edge to maintain directional cell migration (4).

The dynamic remodeling of focal adhesions is regulated by various pathways. One of the best studied regulators of focal adhesion remodeling is the Rho family GTPases, which include RhoA, Rac1, and Cdc42 (5). Exemplar RhoA cycles between the GDP-bound, inactive form and the GTP-bound, active form. Activation of Rho GTPases through GTP binding is catalyzed by guanine nucleotide exchange factors (GEFs)[2] and inactivation of RhoA through GTP hydrolysis is facilitated by GTPase-activating proteins (GAPs). Rho guanine nucleotide-dissociation inhibitors provide additional regulatory mechanisms for Rho activities (6).

Two groups of RhoGEFs with characteristic structural features exist: those that contain the tandem Dbl homology-pleckstrin homology domain and those that contain the DOCK homology domain (6, 7). The p115RhoGEF is a Dbl homology-pleckstrin homology family GEF that contains, at its N terminus, a regulator of G protein signaling domain which has been reported to bind to activated $G\alpha_{13}$ and to function as a GAP for the α subunits of heterotrimeric $G_{12/13}$ proteins (8). Binding to $G\alpha_{13}$ stimulates the GEF activity of p115RhoGEF, leading to Rho activation (9). Although elevated Rho activity is often observed in human cancers, only few RhoGEFs have been reported to contain mutations, such as the rearrangement of Bcr and LARG and the missense mutation of Tiam1 (10). As such, proteins that interact with RhoGEFs could play important roles in the observed Rho hyperactivation in cancers.

β_2-Adrenergic receptors (β_2ARs) are G_s/G_i protein-coupled receptors (GPCRs) and have been implicated in human cancer (11–13). The activation of β_2AR promotes angiogenesis, and growth and invasion of ovarian tumors in xenograft mouse model (14). A role for βARs in cancer initiation is further supported by the observation that long term use of β-blockers is

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[2] The abbreviations used are: GEF, guanine nucleotide exchange factor; β_2AR, β_2-adrenergic receptor; DLC1, deleted in liver cancer 1; GAP, GTPase-activating protein; GPCR, G protein-coupled receptor; ISO, isoproterenol; MEF, mouse embryonic fibroblast; RBD, Rho binding domain of rhotekin; RCC, renal cell carcinoma.

associated with reduced risk of prostate cancer (15), consistent with the observation that stimulated β_2AR transactivates the androgen receptor (16). Mechanistically, activated βARs increase production of matrix metalloproteinase-2 and -9 and vascular endothelial growth factor in nasopharyngeal carcinoma cells (17), promote tumorigenesis through the activation of non-receptor tyrosine kinase c-Src and ERK MAP kinases (18, 19), and mediate stress-induced DNA damage through βArrestin1-dependent suppression of p53 (20).

The best studied β_2AR signaling pathway involves activation of $G\alpha_s$ resulting in the accumulation of intracellular cAMP that activates protein kinase A (PKA). Signaling from β_2AR is dampened by βArrestin1 and βArrestin2 proteins principally by interdicting the receptor coupling to effector heterotrimeric G proteins. The βArrestin proteins also regulate active receptor desensitization and internalization (21). Emerging evidence indicates that these proteins transduce signals independently of heterotrimeric G proteins, providing the basis for the novel concept of biased agonism (22). Thus, a biased ligand can selectively induce the G protein response or βArrestin response (23). For example, the β_2AR antagonist carvedilol stimulates βArrestin2-dependent ERK activation (24) and induces transactivation of EGF receptor that may contribute to the cardioprotective effects of carvedilol (25). Currently, there is little information to incorporate biased agonism into the armamentarium of cancer therapeutics.

In this study, we investigated whether β_2AR regulates focal adhesion remodeling. Acute treatment with β_2AR agonists or βArrestin-biased β_2AR ligands increased cellular content of focal adhesions. Activation of β_2AR induced translocation of p115RhoGEF from the cytosol to the plasma membrane, a process dependent on βArrestin2. Consequent activation of RhoA promoted formation of focal adhesions and stress fibers. These results suggest that βArrestin2 plays cell compartment-specific roles in RhoA activation. In unstimulated cells, βArrestin2 sequesters p115RhoGEF in the cytosol, with lowered net RhoA activation at the plasma membrane. Upon β_2AR activation, βArrestin2 coordinates with p115RhoGEF to activate RhoA at the cell periphery. Therefore, βArrestin2 seems to be an integral part in focal adhesion remodeling elicited by β_2ARs.

EXPERIMENTAL PROCEDURES

Reagents—Anti-FLAG, anti-vinculin, rabbit anti-HA, and anti-actin antibodies were purchased from Sigma; anti-GAPDH from Millipore; mouse anti-HA from Roche Applied Science; anti-p115RhoGEF from Cellular Signaling; anti-RhoA from Cytoskeleton; anti-paxillin from BD Biosciences. Fluorescein isothiocyanate- and rhodamine-conjugated secondary antibodies were from Jackson ImmunoResearch, and rhodamine-conjugated phalloidin was from Invitrogen. siRNAs targeting different genes were from Dharmacon, nontargeting control siRNA from Ambion, and shRNA targeting βArrestin2 from Sigma. Lipofectamine 2000 and Lipofectamine RNAiMax were from Invitrogen, and GenJet from SignaGen. Fetal bovine serum (FBS) was from HyClone; RPMI 1640 medium, DMEM, and penicillin-streptomycin were from Mediatech. Fibronectin and puromycin were from Sigma.

Cell Culture, Transfection, and Immunofluorescence—RCC7 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Mouse embryonic fibroblasts (MEFs) and HEK293 cells were maintained in DMEM supplemented with 10% FBS, penicillin, and streptomycin. Transfection was performed using Lipofectamine 2000 (for cDNAs) or Lipofectamine RNAiMax (for siRNAs) for HEK293 and RCC7 cells, or using GenJet for MEFs. For immunofluorescence staining, cells were seeded on fibronectin-coated coverslips and stained using anti-vinculin, or anti-paxillin (for focal adhesions), anti-HA (for overexpressed βArrestin2), or anti-GFP (for overexpressed GFP-p115RhoGEF) antibodies. Stress fibers were visualized by staining with rhodamine-conjugated phalloidin. Slides were examined using an epifluorescence microscope (DM 6000B; Leica) equipped with a 63×/1.4 −0.6 oil immersion lens, or a Leica Confocal Microscope (TCS SP5; Leica) equipped with a 63×/1.4 NA oil immersion lens. Images were captured and analyzed using the application suite Advanced Fluorescence 2.0.2 software (Leica).

Knockdown of Proteins by siRNA and shRNA—SMARTpool siRNAs targeting βArrestin1, βArrestin2, $G\alpha_i$, or $G\alpha_s$ were purchased from Dharmacon and transfected into cells with Lipofectamine RNAiMax. An equal concentration (100 nM) of nontargeting siRNA was transfected as control. Western blotting was performed 72 h after transfection to examine the efficiency of the knockdown. For stable knockdown of βArrestin2 by shRNA, five lentiviral DNA expression vectors that contained 21 nucleotide shRNA duplex against human βArrestin2 were co-transfected with equal concentrations of vesicular stomatitis virus G and delta8.9 vector into packaging cells. A GFP targeting sequence (5′-GCAAGCTGACCCTGAAGTTCAT-3′) was used as negative control. Virus-containing medium was collected 24 h after transfection and mixed with 5 μl of Polybrene for infection of HEK293 or RCC7 cells. The cells were selected with puromycin (2 μg/ml) 48 h after infection.

Membrane Preparation—The cells were lysed in buffer A containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin A, and 1 mM PMSF. Cells were then disrupted using a Dounce homogenizer with 10 strokes and centrifuged at 1,000 × *g* for 5 min. The supernatant was then centrifuged at 40,000 × *g* for 10 min, and the resulting pellet was washed three times with buffer A. The final pellets were resuspended in a buffer containing 25 mM Tris-HCl, 100 mM NaCl, 1% Triton X-100, and the protease inhibitors described above.

Immunoprecipitation and GST Pulldown—Cells were washed with PBS and lysed in the lysis buffer (25 mM Tris, pH 8.0, 100 mM NaCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin A). Cleared lysates were used for immunoblot or incubated with antibodies overnight for immunoprecipitation, followed by incubation with anti-FLAG M_2 beads for 1 h at 4 °C. Anti-FLAG M_2 beads were washed three times with lysis buffer, and immunoprecipitated proteins were boiled into SDS-PAGE sample buffer. GST fusion proteins were expressed in BL21 cells and purified using glutathione-conjugated agarose affinity medium. The beads with GST fusion pro-

teins bound to them were incubated with freshly prepared cell lysates overnight at 4 °C, washed three times with lysis buffer, and boiled into SDS-PAGE sample buffer. Associated proteins were examined by immunoblotting. Densitometry was performed using Scion Image software.

Statistical Analysis—Data are presented as the mean ± S.E. from at least three independent experiments. Statistical significance was calculated by Student's *t* test or one-way ANOVA with Tukey's post test. Graphs were generated using Prism software (GraphPad), and axis labels were generated using Adobe Illustrator.

RESULTS

β_2AR Regulates Focal Adhesions—G protein-coupled receptors have been shown to regulate cell migration through, at least in part, the regulation of remodeling of actin cytoskeleton (26). Focal adhesion remodeling is an essential part of cell migration and is also regulated by various upstream signals including GPCRs (27). A potential role for βARs in the regulation of focal adhesions has not been reported and is the focus of this study. RCC7 cells, a clear cell renal carcinoma cell line (28, 29), form an average of two to four focal adhesions when plated on fibronectin-coated surface (Fig. 1*A*, *upper panels*). Activation of endogenous βARs with isoproterenol (ISO, 10 μM) resulted in a time-dependent increase in the number of focal adhesions (Fig. 1*B*), as examined by staining with antibodies specific for two different markers of focal adhesions: paxillin and vinculin (Fig. 1*A*, *lower panels*).

ISO is a nonselective agonist that activates all three subtypes of βARs, namely, β_1AR, β_2AR, and β_3AR. We tested the effect of selective βAR agonists on focal adhesions. Treatment of RCC7 cells with either the mixed β_1AR and β_2AR agonist dobutamine, or the selective β_2AR agonist formoterol, increased the number of focal adhesions (Fig. 1*C*), but the selective β_3AR agonist CL316243 was ineffective. These results suggest that β_2AR is the predominant subtype of βARs to regulate focal adhesions.

βARs regulate cellular functions through G protein- and βArrestin-mediated signals (23). As an initial test to differentiate between these signaling pathways, we treated cells with different βAR antagonists. The β_1AR antagonist, CGP21680, and the β_3AR antagonist, SR 59230A, had no effect on the focal adhesion numbers. On the other hand, the β_2AR antagonist, ICI 118,551, and the βAR/α_1AR antagonist, carvedilol, promoted the focal adhesions (Fig. 1*D*). Because both ICI 118,551 and carvedilol are βArrestin-biased ligands on β_2AR, these results suggest that βArrestin-mediated signaling may play a role in the regulation of focal adhesions.

βArrestins Are Involved in Regulation of Focal Adhesions—Next, we examined the role of βArrestins in focal adhesion remodeling induced by β_2ARs. Knockdown of βArrestin1 or βArrestin2 by siRNA (Fig. 2, *A* and *B*) resulted in more focal adhesions (Fig. 2*C*), and ISO treatment failed to significantly further increase the focal adhesion numbers (Fig. 2*C*), as counted by staining with anti-vinculin antibody. This result suggests that βArrestins constitute a critical component in the β_2AR-induced regulation of focal adhesions. Because β_2AR binds βArrestin2 with higher affinity than βArrestin1 *in vivo* (30), we focused on βArrestin2 for further studies.

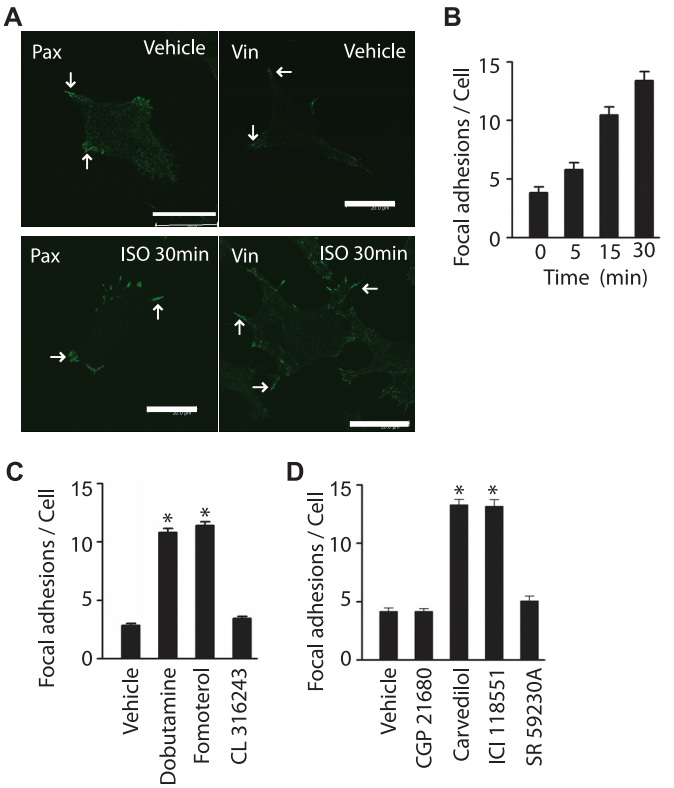
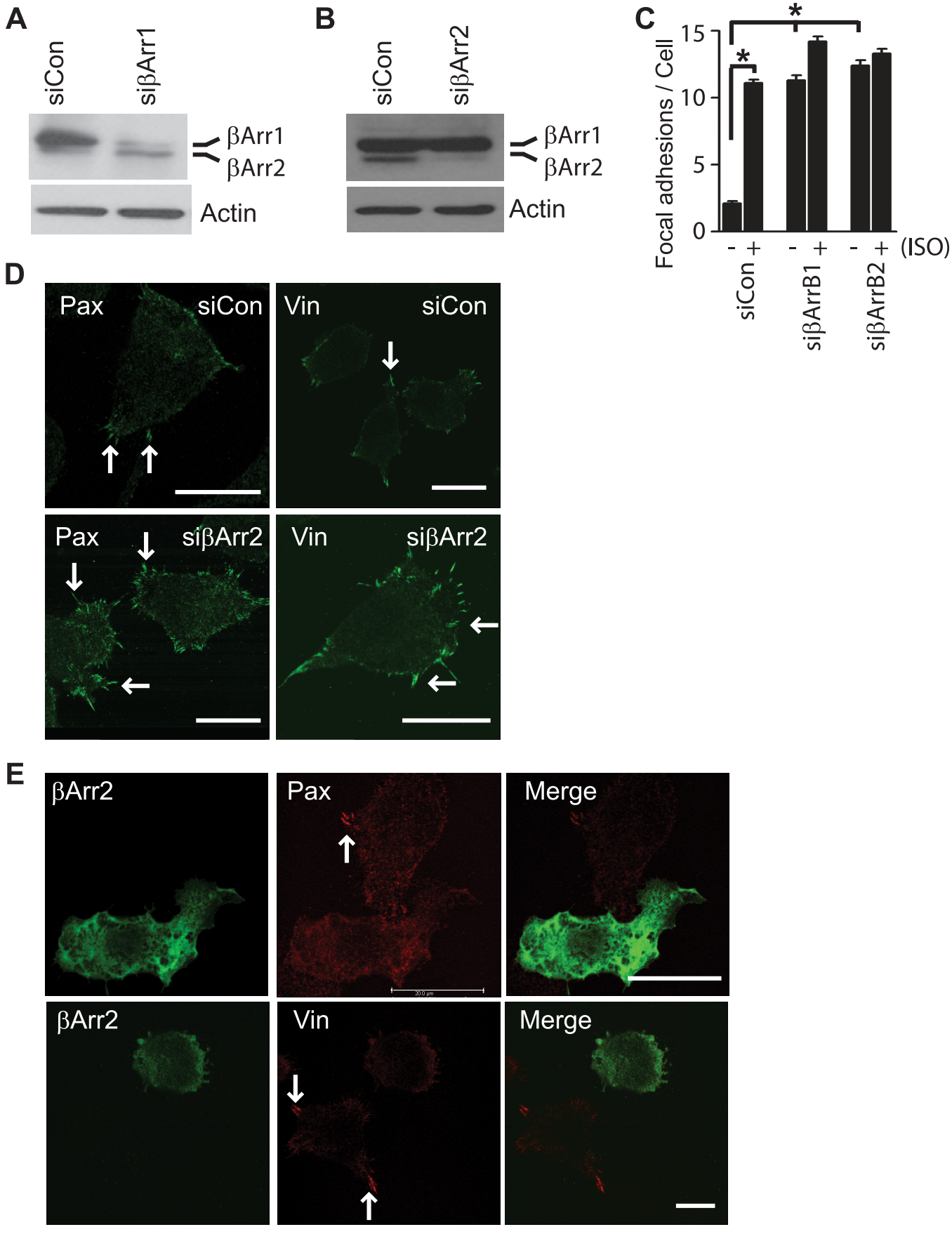


FIGURE 1. **Activation of β_2AR increases focal adhesion numbers in RCC7 cells.** *A*, focal adhesions increased by ISO treatment in RCC7 cells. Cells were plated on fibronectin-coated coverslips in OPTI-MEM for 6 h and treated with vehicle or ISO (10 μM) for 30 min. Cells were fixed, stained with antibodies against paxillin (*Pax*) or vinculin (*Vin*), followed by FITC-conjugated secondary antibody, and examined using confocal microscopy. *Arrows* denote focal adhesions. *Scale bars*, 20 μm. *B*, time course for ISO-induced increase in focal adhesions. RCC7 cells were treated with ISO for the indicated times. Focal adhesions were visualized by staining with anti-vinculin antibody and counted under epifluorescence microscope (Leica DM 6000B). *C*, effect of β_2AR agonists on focal adhesion numbers. RCC7 cells were treated with vehicle, or 1 μM dobutamine, formoterol, or CL316243 for 30 min, and then processed for focal adhesion staining and counting. *D*, effects of βAR antagonists on focal adhesion numbers. RCC7 cells were treated with vehicle, or 1 μM CGP21680, carvedilol, ICI 118,551 or SR 59230A for 30 min before being processed for focal adhesion staining and counting. *, $p < 0.05$ *versus* vehicle. *Error bars*, S.E.

One function of βArrestins is to regulate protein trafficking, and observed alterations in focal adhesion numbers may reflect redistribution of vinculin protein upon knockdown of βArrestins. To exclude this possibility, we examined the formation of focal adhesions by staining with antibodies against paxillin and vinculin, two critical components of focal adhesions. Knockdown of βArrstin2 increased focal adhesion numbers as stained by either anti-paxillin or anti-vinculin (Fig. 2*D*), suggesting that the observed effects are on the focal adhesion structures rather than on the trafficking of a particular component of focal adhesions. As a complementary approach to examine the effect of βArrestin2, we overexpressed epitope-tagged form of the protein. As shown in Fig. 2*E*, overexpression of HA-βArrestin2 disrupted focal adhesions as examined by staining against either paxillin or vinculin.

G_i Is Involved in Regulation of Focal Adhesions by β_2ARs—Because G_s and G_i proteins are the major tranducers of β_2AR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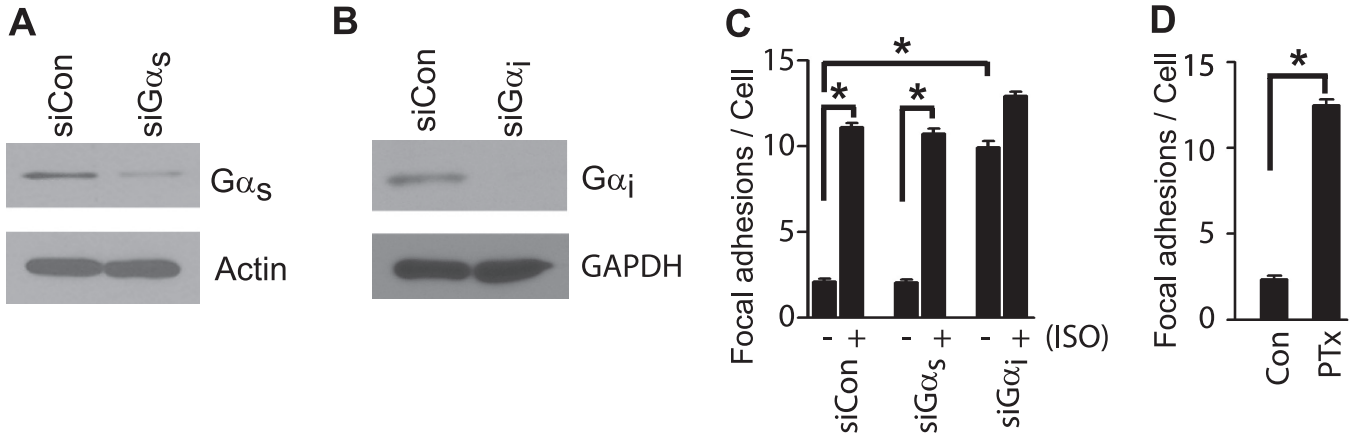


FIGURE 3. **G_i is involved in the regulation of focal adhesions.** *A* and *B*, knockdown of $G\alpha_s$ or $G\alpha_i$ by siRNA. RCC7 cells transfected with control, $G\alpha_s$, or $G\alpha_i$ siRNA were lysed, and the expression of $G\alpha_s$ or $G\alpha_i$ was detected by Western blotting, using actin or GAPDH as loading control. *C*, increased focal adhesion numbers by knockdown of G_i. RCC7 cells were transfected with 100 nM control siRNA or siRNAs targeting the α subunit of G_s or G_i for 72 h. Cells were replated on fibronectin-coated coverslips for 6 h and treated with vehicle or ISO (10 μM) for 30 min before being processed for focal adhesion staining and examination. *D*, increased focal adhesion numbers by inactivation of G_i. RCC7 cells were treated, or not, with pertussis toxin (*PTx*, 75 ng/ml) overnight and replated on fibronectin-coated coverslips in OPTI-MEM in the presence of pertussis toxin for 6 h. Cells were processed for immunofluorescence staining using anti-vinculin antibody. Focal adhesions were counted using epifluorescence microscopy. *, $p < 0.05$; *error bars*, S.E.

signaling(11, 13), we examined potential involvement of these proteins in β_2AR-induced remodeling of focal adhesions. siRNA was used to selectively knockdown expression of $G\alpha_s$ or $G\alpha_i$ (Fig. 3, *A* and *B*). The knockdown of $G\alpha_s$ had no effect on basal or ISO-induced formation of focal adhesions (Fig. 3*C*), suggesting a G_s-independent mechanism in β_2AR-induced focal adhesion remodeling. Remarkably, knockdown of $G\alpha_i$ increased focal adhesion numbers under basal conditions, and ISO treatment only slightly increased the focal adhesion numbers (Fig. 3*C*). To further test the involvement of G_i in the regulation of focal adhesions, we treated cells with pertussis toxin that ADP-ribosylates and inactivates the $G\alpha_i$ proteins. Inactivation of G_i with pertussis toxin increased focal adhesion numbers in RCC7 cells (Fig. 3*D*), reinforcing an active role for G_i proteins in focal adhesion remodeling.

Activation of β_2AR Induces βArrestin2-dependent Activation of RhoA—Focal adhesions are regulated by diverse upstream signals including the RhoA GTPase. To examine whether β_2AR activates RhoA, we used HEK293 cells that express only the β_2 subtype of βARs (20). Activation of Rho GTPases was detected by pulldown assay using GST-RBD (Rho binding domain of rhotekin), or GST-CRIB (Cdc42 and Rac interactive binding domain of PAK), followed by Western blotting using antibodies against RhoA, Rac, and Cdc42. Activation of β_2AR by ISO resulted in the activation of RhoA (Fig. 4*A*), and no consistent activation of Rac or Cdc42 was observed (data not shown). Quantitative densitometry indicated that ISO stimulation increased the RhoA activity by 4-fold (Fig. 4*B*).

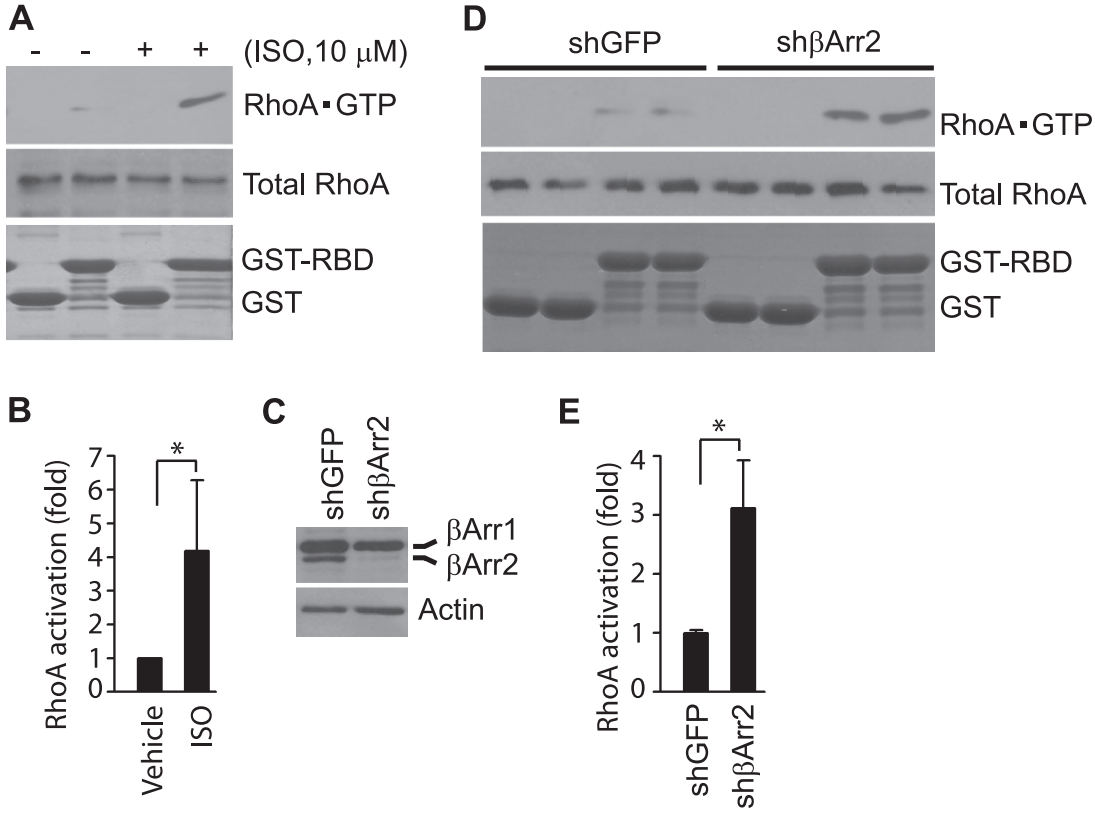
We next examined whether βArrestin2 is involved in the activation of RhoA. For this purpose, we established cell lines with stable knockdown of βArrestin2 using shRNA (Fig. 4*C*). Depletion of βArrestin2 expression (Fig. 4*C*) resulted in an increase in the levels of RhoA·GTP by 3-fold (Fig. 4, *D* and *E*), suggesting that βArrestin2 exerts a tonic inhibition of RhoA. To distinguish the roles of βArrestin1 and βArrestin2 in RhoA activation, we used MEFs from βArrestin1 or βArrestin2 knock-out animals (31). Depletion of cellular βArrestin1 did not change the expression level or activation status of RhoA (Fig. 4*F*). The basal RhoA·GTP levels are almost the same in wild-type and βArrestin1$^{-/-}$ MEFs. ISO (10 μM, 5 min) stimulation increased RhoA activity by 2.2- and 1.8-fold in wild-type and βArrestin1$^{-/-}$ MEFs, respectively (Fig. 4, *F* and *G*). Depletion of βArrestin2 did not affect the expression level of RhoA (Fig. 4*F*), but in βArrestin2$^{-/-}$ MEFs, the basal RhoA·GTP level was approximately 3-fold higher than in βArrestin2$^{+/+}$ MEFs (Fig. 4, *F* and *G*). ISO (10 μM, 5 min) stimulation increased RhoA·GTP levels by 2.4-fold in βArrestin2$^{+/+}$ MEFs, and no further increase in RhoA·GTP level was detected in the βArrestin2$^{-/-}$ MEFs upon ISO stimulation (Fig. 4*G*). These results suggest that βArrestin2 plays a major role in the regulation of RhoA by β_2AR.

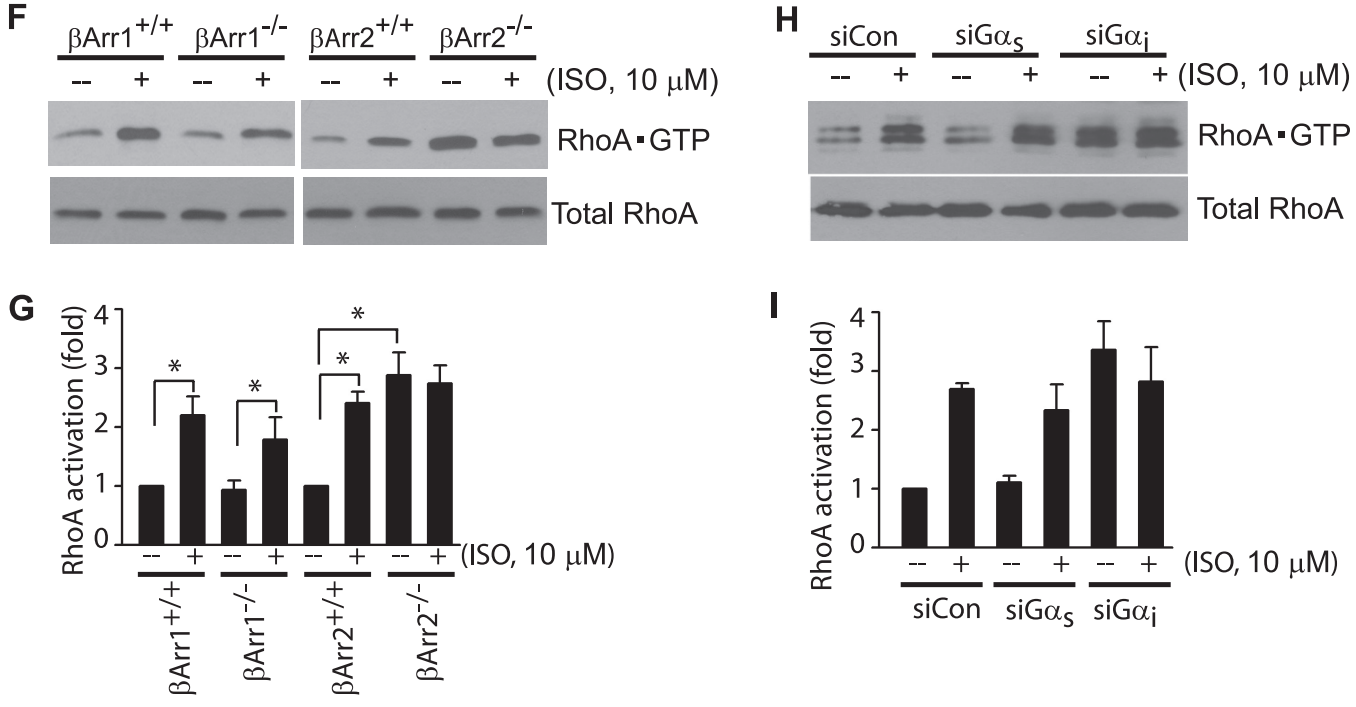
Because our data suggested involvement of G_i proteins in focal adhesion remodeling, we then tested whether G_i regulates RhoA activity. To this end, we knocked down expression of $G\alpha_i$ and $G\alpha_s$, with scrambled siRNA used as a control. Activation of β_2AR with ISO increased RhoA activity in control and $G\alpha_s$

FIGURE 2. **βArrestin2 is involved in β_2AR-induced regulation of focal adhesions.** *A* and *B*, knockdown of βArrestin1 or βArrestin2. RCC7 cells were lysed 72 h after siRNA transfection, and the protein expression level of βArrestin1 or βArrestin2 was examined by Western blotting using A1CT antibody. Level of actin was detected as loading control. *C*, knockdown of βArrestin1 or βArrestin2 effect on focal adhesion formation. RCC7 cells were transfected with 100 nM nontargeting siRNA (*siCon*), siRNA targeting βArrestin1 (*siβArr1*), or βArrestin2 (*siβArr2*) for 72 h. Cells were replated on fibronectin-coated coverslips for 6 h and treated with vehicle or ISO for 30 min. Cells were processed for focal adhesion staining with anti-vinculin antibody and counted under the microscope. *D*, effect of βArrestin2 knockdown on focal adhesion morphology. RCC7 cells transfected with 100 nM control (*siCon*) or βArrestin2 (*siβArr2*) siRNA for 72 h were replated on fibronectin-coated coverslips for 6 h. Cells were stained with antibodies against paxillin (*Pax*) or vinculin (*Vin*), followed by FITC-conjugated goat anti-mouse secondary antibody. Cells were examined using confocal microscopy. *E*, overexpression of βArrestin2 disruption of focal adhesions. RCC7 cells were transfected with cDNAs encoding vector (data not shown) or HA-βArrestin2 for 24 h. Cells were replated on fibronectin-coated coverslips for 6 h and processed for immunofluorescence staining. Focal adhesions were visualized by staining of paxillin or vinculin. Cells overexpressing βArrestin2 were identified by staining against the HA epitope tag. *Arrows* denote focal adhesions. *Scale bars*, 20 μm. *, $p < 0.05$; *error bars*, S.E.

knockdown cells, which exhibited similar level of RhoA activity (Fig. 4, *H* and *I*). Upon $G\alpha_i$ knockdown, however, the basal RhoA activity is significantly increased, and ISO stimulation failed to further activate RhoA (Fig. 4, *H* and I). These results suggest that G_i proteins regulate focal adhesions through RhoA.

βArrestin2 Regulates p115RhoGEF—To indentify intermediates involved in the regulation of RhoA activity by β_2AR and βArrestin2, we screened a selected siRNA library targeting 16 different RhoGEFs on focal adhesion formation. Knockdown of p115RhoGEF, PDZRhoGEF and ArhGEF16 blocked β_2AR-induced increase in focal adhesions (data not shown). We focused on p115RhoGEF for the current study and set out to determine the mechanisms underlying its possible regulation by βArrestin2. First, we examined whether βArrestin2 forms a





complex with the p115RhoGEF. Initial co-immunoprecipitation studies using RCC7 cells were not successful, perhaps due to low transfection rate. Therefore, we used HEK293 cells that are amenable to express higher level of epitope-tagged proteins. Transiently expressed FLAG-tagged βArrestin2 co-immunoprecipitated endogenous p115RhoGEF (Fig. 5*A*), demonstrating that βArrestin2 associates with p115RhoGEF.

The p115RhoGEF is a cytosolic protein, and its activation encompasses translocation to the plasma membrane. Hence, we examined whether β_2AR and βArrestin2 regulate the subcellular distribution of p115RhoGEF. In RCC7 cells, overexpressed GFP-p115RhoGEF and HA-βArrestin2 are diffusely distributed in the cytosol (Fig. 5*B*, *upper panels*). Activation of endogenous β_2AR led to enrichment of both proteins at the plasma membrane, and GFP-p115RhoGEF co-localized with βArrestin2 at the plasma membrane (Fig. 5*B*, *lower panels*).

To study further the role of βArrestin2 in the regulation of p115RhoGEF, we expressed GFP-p115RhoGEF in RCC7 cells with or without knockdown of βArrestin2. In RCC7 cells transfected with control siRNA, ectopically expressed p115RhoGEF is distributed diffusely in the cytosol (Fig. 5*C*, *left panels*). However, knockdown of βArrestin2 by siRNA altered the intracellular distribution of GFP-p115RhoGEF: a substantial fraction of the protein is now enriched at the plasma membrane (Fig. 5*C*, *right panels*). To provide further support for this conclusion, we examined the intracellular distribution of GFP-p115RhoGEF in βArrestin2$^{-/-}$ MEFs. The distribution patterns of GFP-p115RhoGEF in wild-type and βArrestin2 knock-out cells are similar to those seen in the RCC7 cells with or without transient knockdown of βArrestin2. Plasma membrane enrichment of overexpressed p115RhoGEF was observed in 21% of βArrestin2$^{+/+}$ MEFs compared with 65% of βArrestin2$^{-/-}$ MEFs (Fig. 5*D*). Together, these results imply that βArrestin2 may impact the p115RhoGEF activity by sequestering it in the cytosol.

We then examined the distribution of endogenous p115RhoGEF in βArrestin2$^{-/-}$ MEFs by Western blotting. In βArrestin2$^{+/+}$ MEFs, majority of the p115RhoGEF protein is detected in the cytosolic fraction, and activation of endogenous β_2AR with formoterol resulted in translocation of p115RhoGEF from cytosol to the plasma membrane (Fig. 5*E*). However, localization of the p115RhoGEF protein in βArrestin2$^{-/-}$ MEFs is distinct: plasma membrane distribution of the protein appears to be independent of agonist stimulation (Fig. 5*E*), although activation of β_2AR slightly increased membrane expression of the p115RhoGEF (Fig. 5*E*). The total p115RhoGEF protein level is 60% higher in βArrestin2$^{-/-}$ than in βArrestin2$^{+/+}$ MEFs. However, differential partitioning does not explain the 16-fold higher plasma membrane distribution of p115RhoGEF in βArrestin2$^{-/-}$ than in βArrestin2$^{+/+}$ MEFs under unstimulated conditions (Fig. 5*E*), suggesting an active role for βArrestin2 in the regulation of intracellular distribution of p115RhoGEF.

βArrestin2 Expression Impacts Intracellular RhoA Function—To further implicate βArrestin2 in the regulation of intracellular RhoA function, we examined the formation of focal adhesions and stress fibers that are both regulated by RhoA. For this purpose, we used MEFs that support the formation of focal adhesions and stress fibers. Depletion of βArrestin1 exhibited little effect on focal adhesions or stress fibers (Fig. 6, *A–F*). Noticeably, βArrestin1$^{-/-}$ MEFs (Fig. 6, *D–F*) evidenced more protrusions and loss of the unipolar morphology seen in βArrestin1$^{+/+}$ MEFs (Fig. 6, *A–C*). Distinctly, βArrestin2$^{-/-}$ MEFs (Fig. 6, *J–L*) formed more focal adhesions and thicker stress fibers than βArrestin2$^{+/+}$ MEFs (Fig. 6, *G–I*), consistent with elevated RhoA activity following suppression of βArrestin2 expression. To provide direct evidence that RhoA hyperactivity is responsible for the increased formation of focal adhesions in the βArrestin2$^{-/-}$ MEFs, we decreased the RhoA activity by introducing DLC1 (deleted in liver cancer 1), a RhoGAP (32). Forced overexpression of DLC1 (Fig. 6*M*) dramatically reduced the number of focal adhesions (Fig. 6*N*), suggesting that increased focal adhesions in βArrestin2$^{-/-}$ MEFs result from RhoA overactivation.

DISCUSSION

RCC constitutes the majority of kidney cancers, with clear cell being the predominant subtype (33). Despite recent significant advances in the development of targeted therapies, median survival of patients diagnosed with metastatic RCC has only increased by a few months (34). Hence, identification of additional therapeutic targets is essential to improve the overall survival and the progression-free survival of patients suffering from metastatic RCCs. A better understanding of the molecular mechanisms underlying cancer cell migration and invasion may facilitate the identification of novel therapeutic targets. The major finding of the current study is that acute activation of endogenous β_2AR regulates focal adhesion remodeling, a pre-

FIGURE 4. **β_2AR and βArrestin2 regulate RhoA activity.** *A*, β_2AR-induced RhoA activation. HEK293 cells were treated with vehicle or ISO (10 μM) for 5 min, and cleared lysates were used for pulldown assay using GST or GST-RBD of rhotekin. *Top*, RhoA·GTP; *middle*, total RhoA; *bottom*, Coomassie Blue staining of GST or GST-RBD. *B*, quantification of RhoA activity in cells with and without β_2AR activation. Relative active RhoA levels in control and β_2AR-activated cells was determined by Western blotting followed by densitometry. *C*, Western blotting for stable knockdown of βArrestin2. HEK293 cells with stable transfection of shGFP or shβArrestin2 (*shβArr2*) were lysed and processed for Western blotting using A1CT antibody to detect the expression of both βArrestin1 and βArrestin2. Actin was examined for loading control. *D*, stable knockdown of βArrestin2 results in activation of RhoA. HEK293 cells with or without stable knockdown of βArrestin2 were compared for basal RhoA activity by pulldown assay followed by Western blotting. *Top*, active RhoA level by Western blotting; *middle*, total RhoA; *bottom*, Coomassie staining of GST and GST-RBD. Representative images from one of three independent experiments with similar results are shown. *E*, quantification of RhoA activity following stable knockdown of βArrestin2. Relative active RhoA levels in control and βArrestin2 knockdown cells were determined by Western blotting followed by densitometry. *F*, activation of β_2AR failure to increase RhoA activity in βArrestin2$^{-/-}$ MEFs. MEFs with or without βArrestin1 or βArrestin2 knock-out were treated with vehicle or ISO (10 μM, 5 min), and the active RhoA levels were determined by pulldown assay followed by Western blotting. Total RhoA levels were determined to show that depletion of βArrestin1 or βArrestin2 does not affect RhoA protein expression. *G*, quantification of RhoA activity in cells with or without depletion of βArrestin1 or βArrestin2. Active RhoA levels as determined by Western blotting following GST-RBD pulldown were quantified by densitometry. *H*, increased basal RhoA activity by knockdown of G_i. RCC7 cells were transfected with control, $G\alpha_s$, or $G\alpha_i$ siRNA for 72 h. Cells were treated with vehicle or ISO (10 μM, 5 min), and active RhoA levels were measured by pulldown assay followed by Western blotting. *I*, quantification of RhoA activity in cells with or without knock-out of $G\alpha_s$ or $G\alpha_i$. Active RhoA levels as determined by Western blot following GST-RBD pulldown were quantified by densitometry. *, $p < 0.05$; *error bars*, S.E.

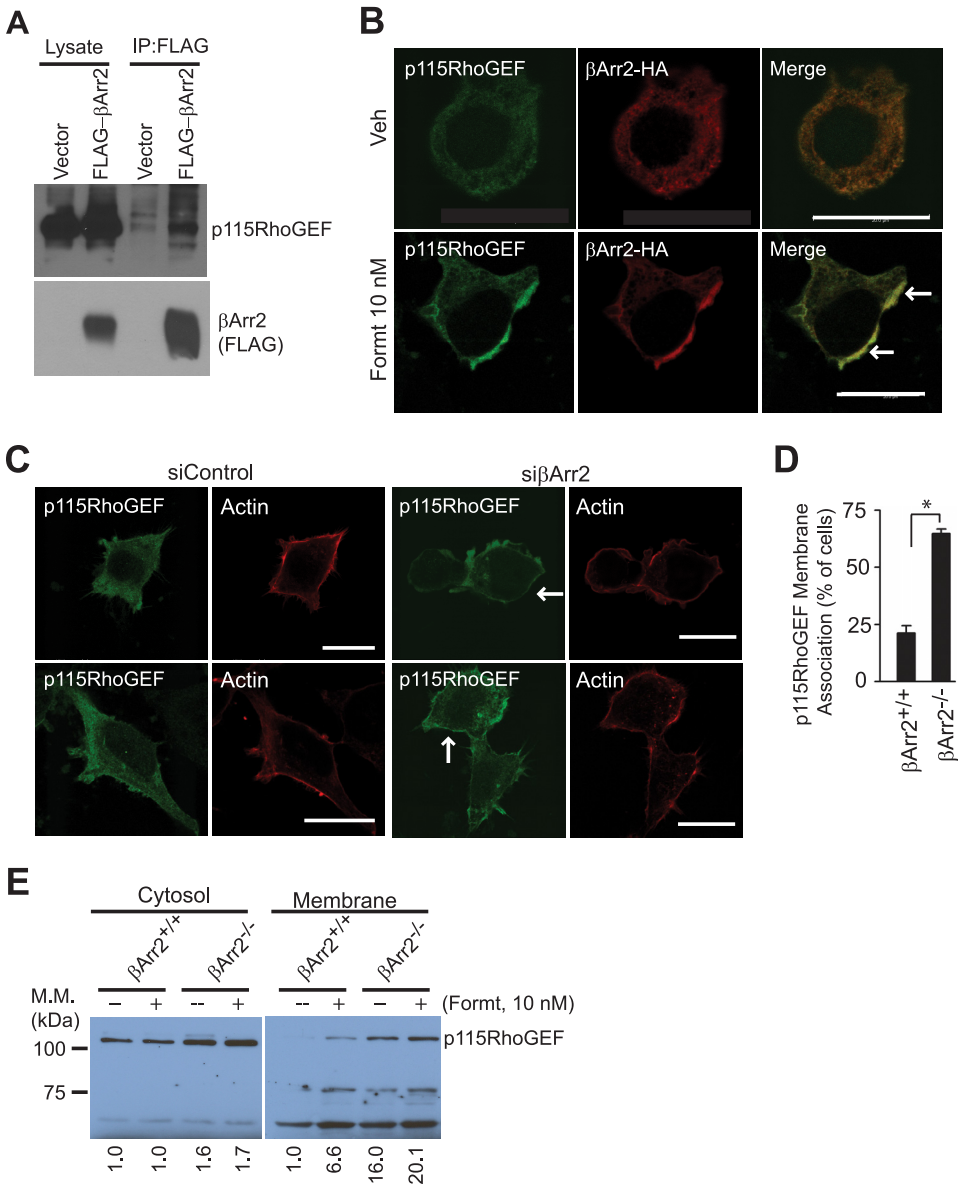


FIGURE 5. **βArrestin2 regulates p115RhoGEF.** *A*, p115RhoGEF formed a complex with βArrestin2. HEK293 cells were transfected with cDNAs encoding empty vector or FLAG-βArrestin2 for 24 h. Cleared cell lysates were used for immunoprecipitation (*IP*) by anti-FLAG antibody, and co-precipitated p115RhoGEF was detected by Western blotting. *B*, βArrestin2 and p115RhoGEF co-localized on plasma membrane upon activation of β_2AR. RCC7 cells were transfected with cDNAs encoding GFP-p115RhoGEF and HA-βArrestin2 for 24 h. Cells were trypsinized and replated on fibronectin-coated coverslips for 6 h and treated with vehicle or formoterol (*Formt*, 10 nM, 5 min). Cells were fixed and processed for immunofluorescence with anti-GFP and anti-HA antibodies. *Arrows* indicate co-localization of p115RhoGEF and βArrestin2 on the plasma membrane. *C*, knockdown of βArrestin2 increased plasma membrane association of p115RhoGEF. RCC7 cells were transfected with 100 nM control or βArrestin2 siRNA for 24 h, then transfected with cDNAs encoding GFP-p115RhoGEF for additional 48 h. Cells were replated on fibronectin-coated coverslips and stained with anti-GFP antibody to visualize overexpressed p115RhoGEF and with rhodamine-conjugated phalloidin to visualize actin cytoskeleton. *Arrows* indicate plasma membrane association of p115RhoGEF. *D*, depletion of βArrestin2 increased p115RhoGEF association with plasma membrane. βArrestin2$^{+/+}$ and βArrestin2$^{-/-}$ MEFs were transfected with cDNA encoding GFP-p115RhoGEF for 24 h and replated on fibronectin-coated coverslips. Cells were processed for immunofluorescence with anti-GFP antibody, and membrane association of GFP-p115RhoGEF was examined by fluorescent microscopy. At least 50 transfected cells were counted from each group in three independent experiments. *E*, increased localization of p115RhoGEF to the plasma membrane in βArrestin2$^{-/-}$ MEFs is shown. βArrestin2$^{+/+}$ and βArrestin2$^{-/-}$ MEFs were treated with vehicle or formoterol (*Formt*) for 5 min. Cells were lysed, and cytosolic and membrane fractions were prepared and subjected to Western blotting using anti-p115RhoGEF antibody. The *numbers* underneath individual *bands* indicate the relative level of p115RhoGEF as determined by densitometry, using levels in the cytosol or membrane in unstimulated βArr2$^{+/+}$ MEFs as arbitrary 1 unit, respectively. *Scale bars*, 20 μm. *, $p < 0.05$; *error bars*, S.E.

requisite for directional cell migration. The effect of β_2AR is mediated through βArrestin2-dependent regulation of p115RhoGEF, a regulator of the $G_{12/13}$ and RhoA activities, which are both implicated in human malignancies (35, 36). Our results indicate that G_i proteins are also involved in the regulation of RhoA and focal adhesions. It is not clear yet whether G_i and βArrestin2 function in tandem or independent of each other for this effect of β_2AR. Nonetheless, the regulation of RhoA activity may provide a way wherein activated β_2AR controls the metastatic dissemination of human RCCs.

G protein-coupled receptors are well known to regulate actin remodeling and cell migration, and the proposed

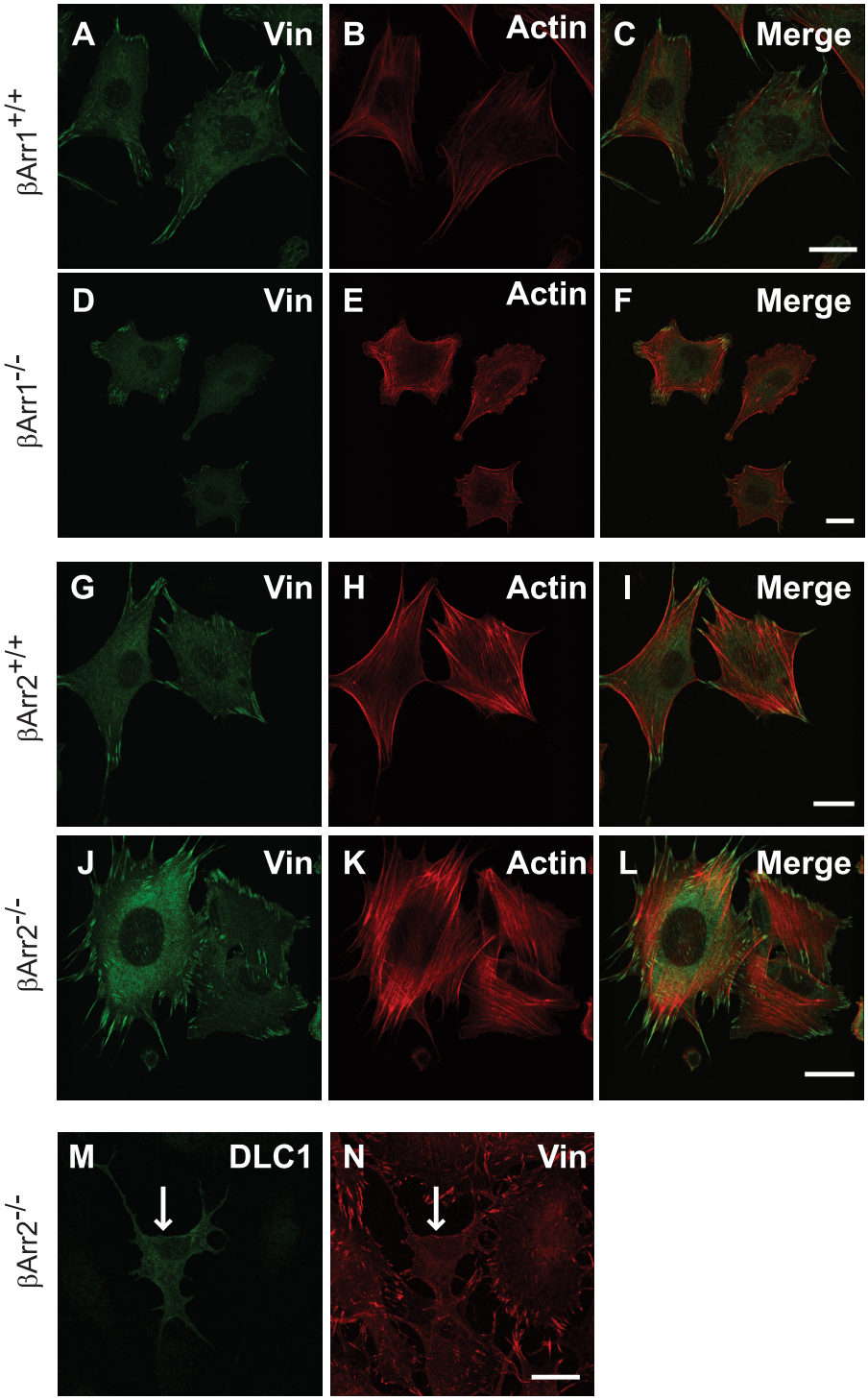


FIGURE 6. **βArrestin2 regulates intracellular RhoA function.** *A–F*, depletion of βArrestin1 does not affect focal adhesions. βArrestin1$^{+/+}$ and βArrestin1$^{-/-}$ MEFs were plated on fibronectin-coated coverslips, and the formation of focal adhesions was examined by staining using anti-vinculin antibody (*A* and *D*), and actin stress fibers stained with rhodamine-conjugated phalloidin (*B* and *E*). *G–L*, depletion of βArrestin2 promoted formation of focal adhesions and stress fibers. βArrestin2$^{+/+}$ and βArrestin2$^{-/-}$ MEFs were examined for the formation of focal adhesions (*G* and *J*) and stress fibers (*H* and *K*). *M* and *N*, overexpressed RhoGAP blocked focal adhesion formation. βArrestin2$^{-/-}$ MEFs were transfected with cDNA encoding the RhoGAP, DLC1 for 24 h and replated on fibronectin-coated coverslips. Cells overexpressing DLC1 were detected by immunofluorescence using anti-DLC1 antibody (*M*), and formation of focal adhesions was examined by immunofluorescence with anti-vinculin antibody (*N*). *Arrows* denote DLC1-overexpressing cells. *Scale bars*, 20 μm.

mechanisms include regulation of kinase activities such as ERK and activation of Rho GTPases (26). The effect of a particular receptor on cell migration may vary depending on the assay conditions or cell types. For example, β_2ARs have been shown to promote the migration of majority of cancer cell types examined (37, 38). However, inhibition of cell migration by β_2ARs has also been observed (39). In glioma cells, for example, activation of β_2AR inhibits lysophosphatidic acid-promoted migration through activation of Epac and Rap1B (40).

Activation of β_2AR was reported to induce cell adhesion to fibronectin through activation of Rap1 (41), and it can be inferred that focal adhesions were actively involved in this process. Focal adhesion remodeling is regulated by many signaling pathways including focal adhesion kinase, Rho GTPases, and mechanical forces (42, 43). The Arf family of GTP-binding proteins is also involved in focal adhesion remodeling. Arf1 was shown to recruit paxillin to focal adhesions (44), and Arf6 is involved in the recycling of β_1-integrin (45). Several Arf GAPs, including GIT1, AGAP2, ARAP2, ASAP1, and ASAP3, are either associated with focal adhesion components or present in focal adhesions (46–50). Both Arf and Arf GAPs may provide a mechanism whereby GPCRs regulate focal adhesion. For example, both βArrestin1 and βArrestin2 bind Arf6 (51), and Arf6 is involved in focal adhesion turnover induced by the endothelin ET_{1b} receptor (27).

Recent studies have provided some insights into the mechanisms underlying βArrestin-mediated regulation of actin remodeling and cell migration. βArrestin1 was reported to mediate the prostaglandin E2-induced activation of c-Src and migration of lung cancer cells (52). The βArrestin-dependent regulation of cell migration may proceed through interactions with the actin-binding protein filamin A (53), the actin-severing protein cofilin (54), or through activation of Cdc42 downstream of TGFβ (55). Our results indicate that RhoA-mediated focal adhesion remodeling is another means by which βArrestins regulate actin cytoskeleton remodeling and cell migration.

At least two possibilities exist for βArrestins to regulate RhoA activity: activation of RhoGEF or inhibition of RhoGAP. Angiotensin II type 1A receptors were shown to promote association of βArrestin1 with the RhoGAP, ARHGAP21. This interaction contributed to the inhibition of the RhoGAP activity of ARHGAP21, leading to higher RhoA activity (56). Combined with the observed activation of RhoGEF by β_2AR and βArrestin2 in the current study, it seems that βArrestins fine-tune RhoA activity through coordinated regulation of RhoA activators and inactivators.

$G_{12/13}$ are principal activators of p115RhoGEF downstream of GPCRs (35). It was reported that the $G_{q/11}$-coupled angiotensin II type 1A receptor activates RhoA (57), albeit by as yet undetermined mechanism(s). A recent study suggested that angiotensin II elicits G_q-dependent and JAK2-mediated phosphorylation and activation of p115RhoGEF (58). Our results provide evidence that GPCRs with no known coupling to these heterotrimeric G proteins could also impact the RhoA activity through βArrestin2-dependent activation of p115RhoGEF. As βArrestins function as "universal" signal regulators for GPCRs, it is reasonable to predict that additional receptors that do not couple to $G_{12/13}$ or G_q are endowed with the ability to activate the p115RhoGEF. Hence, βArrestins may serve as a convergence point for diverse upstream signals to activate RhoA. For example, angiotensin II type 1A receptor-induced activation of RhoA required both G_q and βArrestin1 (57). At present, the potential involvement of G_i in β_2AR-induced activation of p115RhoGEF cannot be ruled out. Consistent with this notion, we observed the G_i-mediated regulation of RhoA and focal adhesions. This is also in agreement with previous findings that G_i is involved in plasma membrane translocation of RhoA (59).

Several mechanisms have been described to regulate the p115RhoGEF activity, including $G_{12/13}$-mediated membrane translocation that also requires the pleckstrin homology domain, C termini oligomerization-induced inhibition, autoinhibition by the linker region, and phosphorylation by protein kinase C (60–63). Our results provide support for a new model in which βArrestin2 sequesters p115RhoGEF in the cytosol in its inactive state, and p115RhoGEF translocates to the plasma membrane upon βArrestin2-biased ligand binding to β_2AR. It is well established that β_2AR activation results in membrane translocation of βArrestin2. Therefore, p115RhoGEF may co-translocate to the plasma membrane with βArrestin2 upon agonist stimulation of β_2AR. Alternatively, p115RhoGEF may dissociate from βArrestin2 upon β_2AR activation and gets targeted to the plasma membrane by as yet undetermined mechanism. The knowledge that β_2AR couples to G_s and G_i but not $G_{12/13}$, together with our finding that knockdown of $G\alpha_s$ does not impact β_2AR-induced focal adhesion remodeling, supports existence of binding partner(s) other than G_{13} for the p115RhoGEF distribution on the plasma membrane. Based on our observations that p115RhoGEF co-localizes with βArrestin2 on the plasma membrane upon β_2AR activation, it is feasible that βArrestin2 facilitates the plasma membrane expression of p115RhoGEF.

In the case of βArrestin2 knockdown, the inhibitory effect of βArrestin2 on p115RhoGEF in the cytosol is relieved. As a result, p115RhoGEF translocates to plasma membrane, leading to activation of RhoA and formation of focal adhesions. Under this condition, β_2AR activation is no longer a prerequisite for βArrestin2 membrane association. A second, yet not exclusive, possibility is that knockdown of βArrestin2 reduces desensitization of certain GPCRs which normally exhibit basal activities, and the consequently elevated receptor signaling results in activation of RhoA and focal adhesion formation.

PKA is reported to phosphorylate RhoA thus decreasing RhoA binding to its effector kinase ROCK (64). In addition, phosphorylation of RhoA by PKA results in the association of RhoA with Rho GDP-dissociation inhibitor and consequent translocation of active RhoA·GTP to the cytosol (65). Hence, two distinct mechanisms may exist for the β_2AR to inhibit activity of RhoA·GTP: decreased coupling to its effector kinase ROCK and lowered distribution on the plasma membrane. Here, we showed that β_2AR can activate RhoA through p115RhoGEF. Combined, these results suggest that β_2AR modulates RhoA activity through both feed-forward activation and feedback inhibition loops.

In summary, we have provided evidence that acute activation of β_2AR promotes the βArrestin2-dependent activation of p115RhoGEF and RhoA, leading to enhanced formation of focal adhesions. In unstimulated cells, cytosolic βArrestin2 may prevent membrane translocation and activation of p115RhoGEF by sequestering it in the cytosol. Therefore, βArrestin2 may exert cell compartment-specific regulation of RhoGEF and RhoA activities. Because spatial and temporal regulations of RhoA activities are critical for directed cell migra-

tion, βArrestin2 may play deciding roles in the regulation of cell migration by β_2ARs.

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