Supporting Information

Oakley et al. 10.1073/pnas.1209411109

SI Materials and Methods

Reagents. Dexamethasone, cortisol, corticosterone, and mifepristone (RU486) were purchased from Steraloids. Cycloheximide and actinomycin D were purchased from Sigma-Aldrich. The PAR1-activating peptide, TFLLR-amide, was obtained from Bachem. The anti–β-arrestin antibody was purchased from Novus Biologicals, and the anti–β-actin antibody was purchased from Millipore. Generation of the rabbit polyclonal anti-GR 57 antibody has been described previously (1). The anti-phosphorylated ERK and anti-ERK antibodies were obtained from Cell Signaling Technology. The anti-PAR1 antibody (clone WEDE15) was purchased from Beckman Coulter, and the anti-FLAG M2 antibody was purchased from Sigma-Aldrich.

Cell Culture. A549 and U2OS cells (American Type Culture Collection) were maintained in DMEM/F-12 (Invitrogen) supplemented with 10% FCS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine. Mouse embryonic fibroblasts, kindly provided by Dr. Robert J. Lefkowitz (Duke University), were maintained DMEM/high glucose (Invitrogen) with 10% FCS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine. The generation of U2OS cells stably expressing the human GR has been described previously (2). Before treatments, cells were cultured in medium containing HyClone charcoal/dextran-stripped FCS (Thermo Scientific).

RNA Isolation and Quantitative RT-PCR Analysis. Total RNA was isolated from cells using the RNeasy Mini Kit and RNase-Free DNase Kit (Qiagen) according to the manufacturer's protocol. The abundance of individual mRNAs was determined using a Taqman one-step RT-PCR procedure on a 7900HT sequence detection system (Applied Biosystems). Predeveloped primer/probe sets for β-arrestin-1, β-arrestin-2, PAR1, and cyclophilin B were obtained from Applied Biosystems. Values measured for each primer/probe set were normalized to the housekeeping gene peptidylprolyl isomerase B (cyclophilin B), which is not regulated by glucocorticoids. For analysis of nascent RNA, primer sequences were designed to amplify regions spanning an exon–intron boundary, thereby permitting the detection of only unprocessed, newly expressed transcripts. These primer sequences were as follows: for β-arrestin-1: forward primer, 5′-TACCTGGGAAAGCGGACTTTGT-3′ (exon 3); probe, 5′-ACCACATCGACCTCGTGGACCCTGT-3′; reverse primer, 5′-TGAGAGCTATTCTGGGAGGCTTG-3′ (intron 3); for β-arrestin-2: forward primer, 5′-AAACCACACGCCACTTCCTCATGT-3′ (exon 8); probe, 5′-TGACACGGATGCCACGGTGCAGTTTA-3′; reverse primer, 5′-CAGCCAAGGCAACCTCCTTTACAA-3′ (intron 8); for cyclophilin B: forward primer, 5′-ATGAAGATGTAGGCCGGGTGATCT-3′ (exon 2); probe, 5′-TTTGTGGCCTTAGCTACAGGAGAGGT-3′; reverse primer, 5′-TGAACTCTGCAGGTCAGTTTGCTG-3′ (intron 2).

Immunoblot Analysis. Cells were washed once with cold PBS on ice, lysed in SDS sample buffer (Invitrogen) supplemented with β-mercaptoethanol, sonicated on ice, and boiled for 5 min. Total protein was determined using the Pierce 660-nm protein assay with an ionic detergent compatibility reagent (Thermo Scientific). Equivalent amounts of protein were resolved on 8% Tris-glycine gels (Invitrogen) and transferred to nitrocellulose. After blocking for 1 h, membranes were incubated overnight at 4 °C with primary antibodies to β-arrestin-1, β-arrestin-2, GR, phosphorylated ERK, total ERK, PAR1, or β-actin. Blots were washed and incubated with goat anti-rabbit Alexa Fluor 680-conjugated secondary antibody (Invitrogen) and/or goat anti-mouse IRDye800-conjugated secondary antibody (Rockland Immunochemicals) for 1 h at room temperature and developed using the LI-COR Odyssey imaging system. Levels of β-arrestin-1 and β-arrestin-2 were quantified and normalized to β-actin. For some experiments, blots were incubated with HRP-linked secondary antibodies and developed using enhanced chemiluminescence (GE Healthcare).

Animal Studies. Adrenalectomized C57BL/6J male mice were purchased from Charles River Laboratories and treated by i.p. injection with vehicle (PBS) or 1 mg/kg Dex. Animals were killed by cervical dislocation, and tissues were harvested for RNA or protein extraction. Tissues used for RNA extraction were incubated overnight in RNAlater (Qiagen) and processed for RT-PCR analysis as described above. Tissues used for protein extraction were immediately homogenized in SDS sample buffer (Invitrogen) supplemented with β-mercaptoethanol using a Tissue-mizer, boiled for 5 min, centrifuged to remove debris, and processed for immunoblot analysis as described above. These studies were approved by the National Institute of Environmental Health Sciences' Animal Care and Use Committee.

ChIP Assays. ChIP assays were performed as described previously with minor modifications (3). In brief, A549 cells were fixed in 1% paraformaldehyde for 10 min at room temperature and harvested in cell lysis buffer containing protease inhibitors (Millipore). Nuclei were collected and resuspended in shearing buffer [10 mM Tris-HCl (pH 8), 1 mM EDTA, 140 mM NaCl, 1% SDS, 0.1% Na deoxycholate, 1% Triton X-100, protease inhibitor mixture]. DNA was sheared by sonication using a Branson Sonifier 150 on setting 4. Protein–DNA complexes were immunoprecipitated overnight with equivalent amounts of IgG or anti-GR antibodies. Immunoprecipitated protein–DNA complexes were retrieved with protein A magnetic beads (Millipore), washed, and eluted. After the cross-links were reversed, the DNA was purified using a QIAquick DNA purification kit (Qiagen). Quantitative RT-PCR was performed on both input and immunoprecipitated DNA using the following primers and probes: forward primer, 5′-AGATCTCTGAGCCCTGCAGAAACA-3′; probe, 5′-AGGAGGGTTCTGGGCTTTCATCCATA-3′; and reverse primer, 5′-ACCTCTGGGCAATGTTAGGAGCTA-3′ to the promoter of β-arrestin-1; forward primer, 5′-AACTGCTTGTTCCTTCCCAGGAGA-3′; probe, 5′-CCCTGGGAGTTGGACAAGACAGGT-3′; and reverse primer, 5′-TTCAGTTCCTGCGGTGACCTTAGT-3′ to intron-1 GRE of β-arrestin-1; forward primer, 5′-AATAGATGGGAGGGCACATGCAGA-3′; probe, 5′-TGGAAGTCTTTCCAGGAGCAACGTGT-3′; and reverse primer, 5′-TGACTAAGGGTGGAGGAAGGCAAA-3′ to the distal promoter of GILZ; forward primer, 5′-CACCGCCACTGAGAAAGCAAACAA-3′; probe, 5′-CCTGCAGGTTCTGGATGCCGAA-3′; and reverse primer, 5′-GGGATCTAAGTCTTTCGATTGGC-3′ to the promoter of β-arrestin-2; and forward primer, 5′-CCAGCTTCACAAAGGGCTGGATTT-3′; probe, 5′-TGCCTTTGTTCAGGGTTCTCACTGCA-3′; and reverse primer, 5′-AAAGCTGACGTCCTTCTCCGCTAA-3′ to the intron-11 nGRE of β-arrestin-2.

Plasmid Constructs. The β-arrestin-1 and β-arrestin-2 genes were searched for GREs and nGREs using Sequence Motif Search (www.genome.jp) and DNA Pattern Find (4). Fragments from

the β-arrestin-1 promoter, β-arrestin-1 intron-1, β-arrestin-2 promoter, and β-arrestin-2 intron-11 were amplified from A549 cell genomic DNA using the FastStart High-Fidelity PCR System (Roche). The β-arrestin-1 promoter (~1.7 kb) was cloned into pGL3-Basic (Promega) to generate βarr1-Pro using the following primers: 5′-GTTCAGGCTAGCGTCCGCGACGGTCGCAG-GGAGGTC-3′ and 5′-GTTCAGGGTACCGTGGAGAGTGG-CAGGACCTGGCTTCACC-3′. The β-arrestin-2 promoter (~1.0 kb) was cloned into pGL3-Basic to generate βarr2-Pro using the following primers: 5′-GTTCACGGTACCTTCTGCG-CATCCTTCAGAAAGACTGTCCTC-3′ and 5′-GTTCACA-GATCTTCGGTTCGCGGCTCGCTCGCAGC-3′. The β-arrestin-1 intron-1 fragment (~500 bp) was cloned into pGL4.23 (Promega) in the forward and reverse orientations to generate intron1F-GRE and intron1R-GRE, respectively, using the following primers: 5′-GTTCAGGGATCCTGCAGTCCCTCC-TATGTGGACAGG-3′ and 5′-GTTCAGGGATCCGCTGTT-GAACCACTGAATGG-3′. The intron-1 GRE sequence GGA-ACAnnnAGTTCT was mutated to GGAAGGnnnAAGTAT using the QuikChange site-directed mutagenesis kit (Stratagene) to generate intron1F-mut and intron1R-mut. The β-arrestin-2 intron-11 fragment (~800 bp) was cloned into pGL4.23 in the forward and reverse orientations to generate intron11F-nGRE and intron11R-nGRE, respectively, using the following primers: 5′-GTTCGTA-GATCTCAGCAGGTTTAGTGACAAGTATGAAGGAG-3′ and 5′-GTTCGTAGATCTGAGAGGCATCGTGAAGAGGACG-ACGAC-3′. The β-arrestin-2 intron-11 fragment (~800 bp) was subcloned into βarr2-Pro in the forward and reverse orientations to generate Pro-intron11F-nGRE and Pro-intron11R-nGRE, respectively. The intron-11 IR1nGRE sequence TTCCnGGA-GA was mutated to TGAAnAAAGA using site-directed mutagenesis to generate Pro-intron11F-mut1. The intron-11 IR1n-GRE was mutated as above, and the IR0nGRE sequence ATCCGGAGA was mutated to AGAAAAAGA using site-directed mutagenesis to generate Pro-intron11F-mut2. The sequence of all constructs was confirmed by DNA sequencing.

Luciferase Assays. A549 cells were transfected in six-well plates using Transit-LT1 (Mirus) with the firefly luciferase reporters described above and a *Renilla* luciferase reporter for assessment of transfection efficiency (2). The day after transfection, cells were harvested and replated at equal densities in a 48-well plate. After an overnight incubation (~18 h) with vehicle or 100 nM Dex, the cells were lysed in passive lysis buffer, and luciferase activity was measured using the Dual Luciferase Reporter Assay (Promega).

Inositol Phosphate Assays. Inositol phosphates were measured essentially as described previously (5). In brief, A549 cells were pretreated with vehicle or 100 nM Dex for 42 h. During the last ∼18 h of this preincubation, cells were labeled with 2.5 μCi/mL of [myo-^{3}H]inositol (Perkin-Elmer) in serum- and inositol phosphate-free DMEM (MP Biomedicals). The PAR1- activating peptide TFLLR was added to the cells for various times in the presence of 10 mM $LiCl_2$. The samples were quenched with perchloric acid and neutralized with potassium carbonate. [^{3}H] Inositol phosphates were separated by anion-exchange chromatography (AG1-X8 resin, Bio-Rad), quantified by scintillation counting, and normalized to the level of [^{3}H] incorporated into total inositol lipids, which were extracted from cell debris using 0.1 N NaOH and 0.1% Triton X-100.

RNA Interference Assays. Nontargeting control siRNA, GR SMARTpool siRNA, and β-arrestin-1 SMARTpool siRNA were purchased from Thermo Scientific. A549 cells were transfected with 60 nM of each siRNA using Dharmafect1 transfection reagent (Thermo Scientific) according to the manufacturer's instructions. The day after transfection, cells were harvested and replated in appropriate tissue culture dishes. Cells were harvested at 72 h posttransfection for isolation of RNA (RT-PCR analysis), preparation of protein lysates (immunoblotting), and measurement of inositol phosphates.

Colocalization Assays. A549 cells were transiently transfected with FLAG-PAR1, kindly provided by Dr. JoAnn Trejo (University of California at San Diego). The cells were then pretreated with vehicle or 100 nM Dex for ∼42 h. During the last 1 h of this preincubation, the cells were cultured in serum-free medium. Either vehicle or the PAR1-activating peptide TFLLR (100 μM) was then added to the cells. After a 5-min incubation, the cells were washed with cold PBS, fixed with 4% paraformaldehyde, and permeabilized with PBS containing 0.1% Triton X-100 and 2% BSA. The cells were then incubated overnight at 4 °C with primary antibodies for β-arrestin and FLAG-PAR1. After washing, the cells were incubated with goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 594 secondary antibodies (Invitrogen). Images were obtained sequentially on a laser scanning confocal microscope (LSM 710; Carl Zeiss) using dual excitation (488 nm from an argon laser, 561 nm from a diode laser) and emission filters (493–591 nm and 591–734 nm). Microscope settings were identical for all images.

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2. Lu NZ, Cidlowski JA (2005) Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. *Mol Cell* 18: 331–342.
3. Nissen RM, Yamamoto KR (2000) The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. *Genes Dev* 14:2314–2329.
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5. Kirk CJ, Morris AJ, Shears SB (1990) Inositol phosphate second messengers. *Peptide Hormone Action: A Practical Approach*, eds Siddle K, Hutton JC (Oxford Univ Press, New York), pp 151–184.

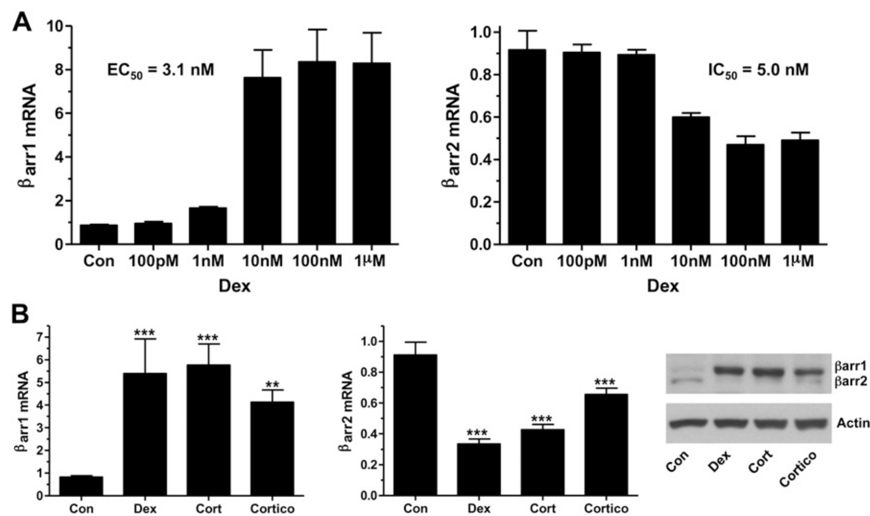

Fig. S1. Glucocorticoid regulation of β-arrestin-1 and β-arrestin-2 gene expression is dose-dependent and occurs with the natural glucocorticoids cortisol and corticosterone. (*A*) A549 cells were treated for 4 h (β-arrestin-1) or 12 h (β-arrestin-2) with vehicle (Con, control) or the indicated concentrations of Dex. The levels of β-arrestin-1 and β-arrestin-2 mRNA were analyzed by RT-PCR. Data represent mean ± SD from between two and three independent experiments. (*B*) A549 cells were treated with vehicle, 100 nM Dex, 500 nM cortisol (Cort), or 1 μM corticosterone (Cortico) for 4 h (β-arrestin-1) or 24 h (β-arrestin-2). (*Left* and *Middle*) Levels of β-arrestin-1 and β-arrestin-2 mRNA measured by RT-PCR. (*Right*) Representative immunoblots of β-arrestin-1 and β-arrestin-2 after a 48-h ligand treatment. Data represent mean ± SD from four independent experiments. $**P < 0.01$; $***P < 0.001$.

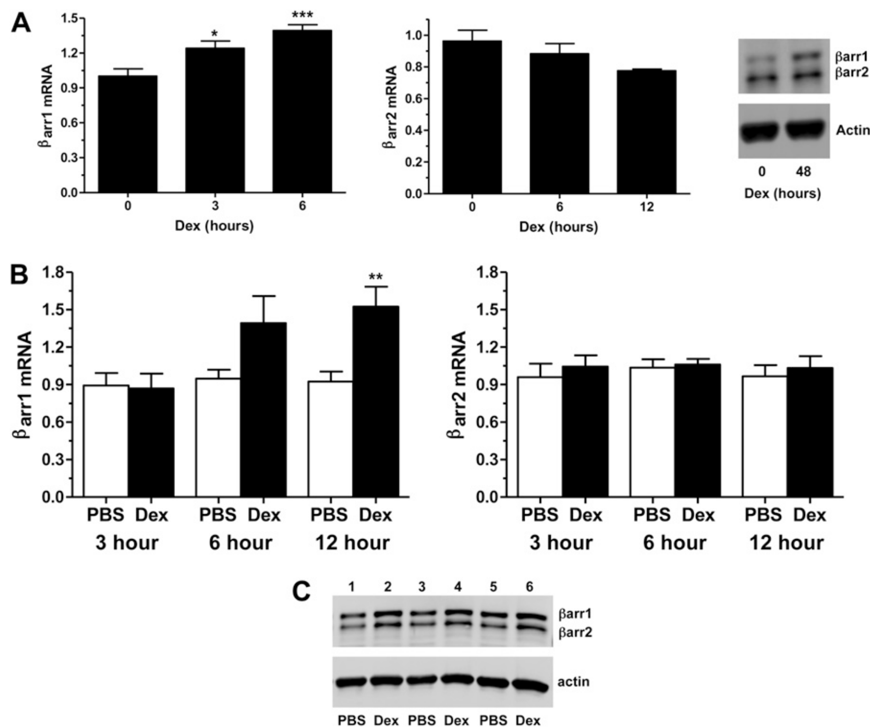

Fig. S2. Glucocorticoids regulate β-arrestin-1 gene expression in mouse embryonic fibroblasts and in vivo in mouse spleen. (*A*) Mouse embryonic fibroblasts were treated with 100 nM Dex for indicated times, and β-arrestin-1 and β-arrestin-2 were evaluated by RT-PCR (*Left* and *Middle*) and immunoblot analysis (*Right*). Data represent mean ± SD from three independent experiments. $*P < 0.05$; $***P < 0.001$. (*B*) Adrenalectomized C57BL/6J mice were injected with vehicle (PBS) or 1 mg/kg Dex for the indicated times. The whole spleen was removed, RNA was isolated, and β-arrestin-1 mRNA (*Left*) and β-arrestin-2 mRNA (*Right*) were analyzed by RT-PCR. Data represent mean ± SEM; $n = 5$ mice per group. $**P < 0.01$. (*C*) Adrenalectomized C57BL/6J mice were injected with vehicle (PBS) or 1 mg/kg Dex once every 12 h; $n = 3$ mice per group. At 12 h after the last injection (total treatment time, 48 h), the entire spleen was removed, protein lysates were prepared, and β-arrestin-1 and β-arrestin-2 protein expression was evaluated by immunoblot analysis.

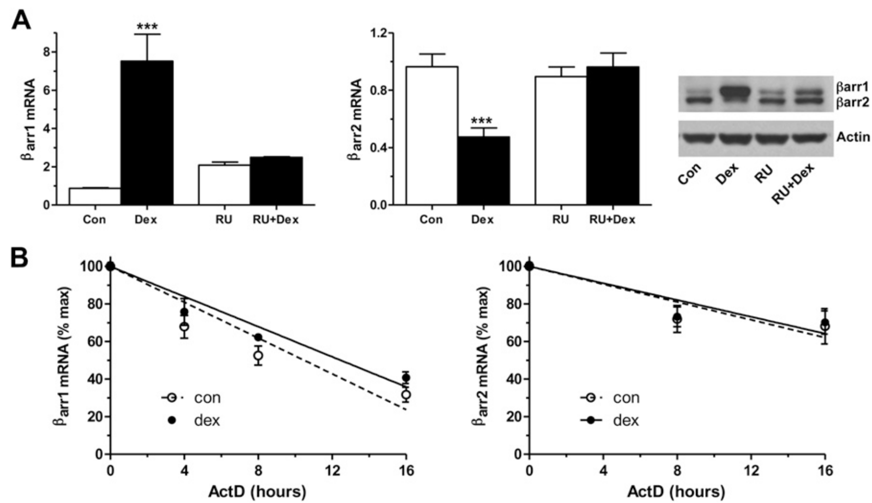

Fig. S3. Glucocorticoid regulation of β-arrestin-1 and β-arrestin-2 gene expression is blocked by RU486 and does not involve significant changes in mRNA half-life. (*A*) A549 cells were treated with vehicle (Con, control) or 100 nM Dex for 4 h (β-arrestin-1) or 12 h (β-arrestin-2) in the presence or absence of 1 μM RU486. (*Left* and *Middle*) Levels of β-arrestin-1 and β-arrestin-2 mRNA were analyzed by RT-PCR. (*Right*) Representative immunoblots of β-arrestin-1 and β-arrestin-2 after a 48-h ligand treatment. Data represent mean ± SD from four independent experiments. ***$P < 0.001$. (*B*) A549 cells pretreated with vehicle or 100 nM Dex for 4 h (β-arrestin-1) or 12 h (β-arrestin-2) were exposed to 5 μg/mL of actinomycin D (ActD) for the indicated times. The levels of β-arrestin-1 mRNA (*Left*) and β-arrestin-2 mRNA (*Right*) were analyzed by RT-PCR. Data represent mean ± SD from three or four independent experiments.

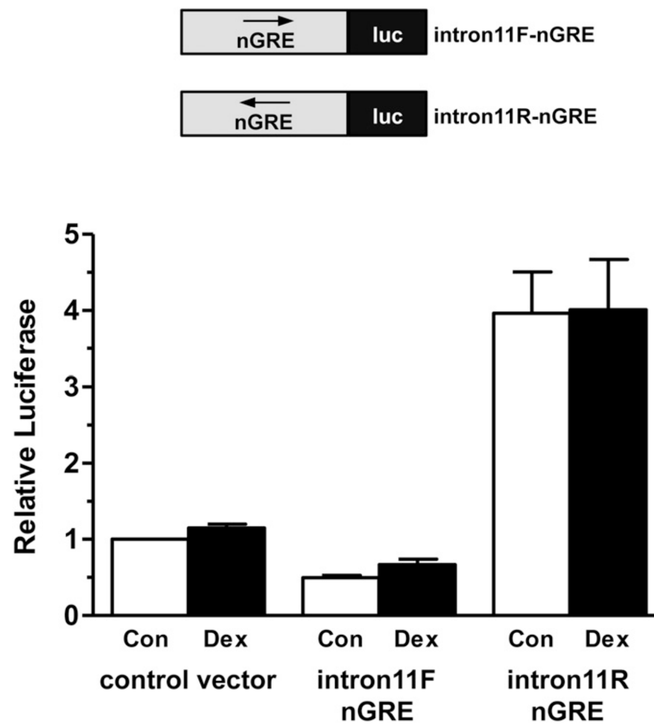

Fig. S4. The ~800-bp intron-11 fragment from the β-arrestin-2 gene is inactive or stimulates luciferase expression only weakly and is unaffected by glucocorticoids. A549 cells were transfected with the indicated luciferase reporter plasmids and treated for ~18 h with vehicle (Con, control) or 100 nM Dex. Cells were then harvested, and luciferase activity was measured. Data represent mean ± SEM from five independent experiments.

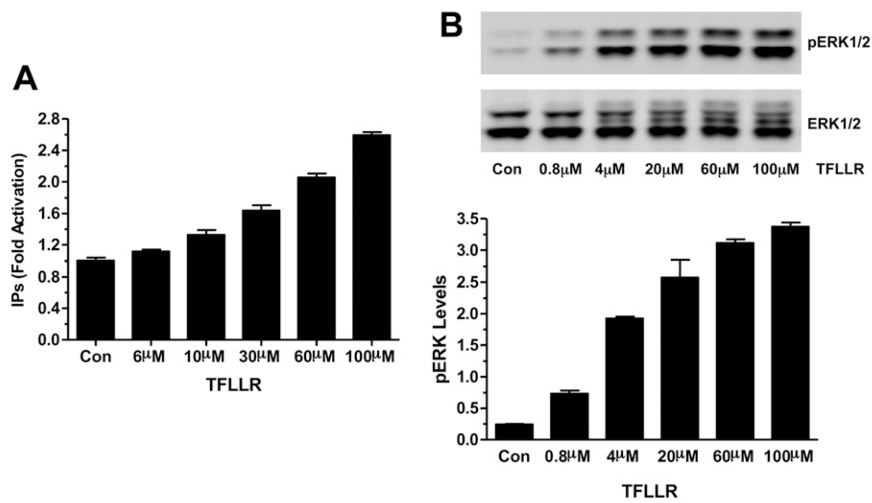

Fig. S5. PAR1 stimulation of inositol phosphate and MAPK-signaling pathways. (*A*) A549 cells were treated for 1 h with the indicated concentrations of the PAR1-activating peptide TFLLR, and total [^{3}H]inositol phosphates (IPs) were measured. Data were normalized to total lipids and plotted as fold increase over basal levels. (*B*) A549 cells were treated for 5 min with the indicated concentrations of the PAR1-activating peptide TFLLR, and phosphorylated ERK was measured by immunoblot analysis. (*Upper*) Representative immunoblot of phosphorylated ERK and total ERK. (*Lower*) Quantitation of phosphorylated ERK normalized to total ERK. Data represent mean ± SD from two or three independent experiments. Con, control.

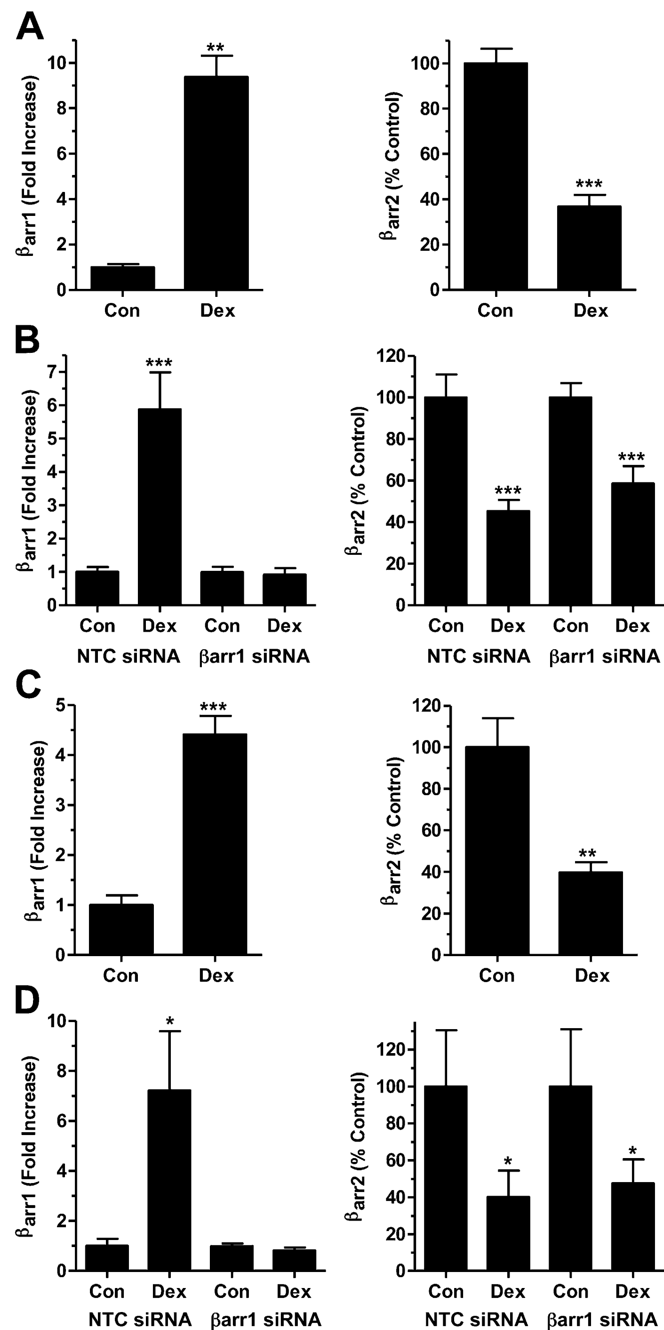

Fig. S6. Glucocorticoid-dependent induction of β-arrestin-1 and repression of β-arrestin-2 protein. Shown is the quantitation of β-arrestin-1 and β-arrestin-2 levels, normalized to actin, in the control (Con) and Dex-treated samples from immunoblots shown in Fig. 5*A* (*A*), Fig. 5*B* (*B*), Fig. 6*A* (*C*), and Fig. 6*B* (*D*). Control values were set to 1.0 for β-arrestin-1 and 100% for β-arrestin-2. Data represent mean ± SEM from three or four independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

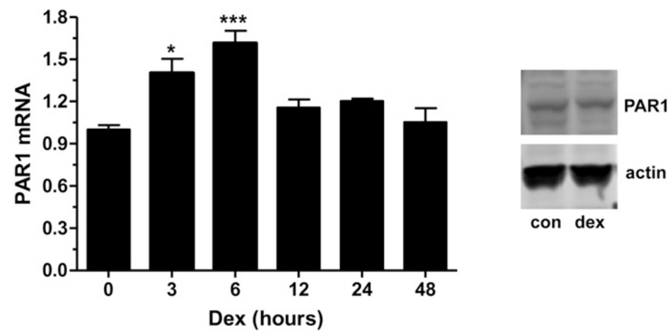

Fig. S7. Glucocorticoid regulation of PAR1 gene expression in A549 cells. A549 cells were treated with 100 nM Dex for the indicated times, and PAR1 levels were evaluated by RT-PCR (*Left*) and immunoblot analysis (*Right*). Data represent mean ± SD from four independent experiments. *$P < 0.05$; ***$P < 0.001$. con, control.

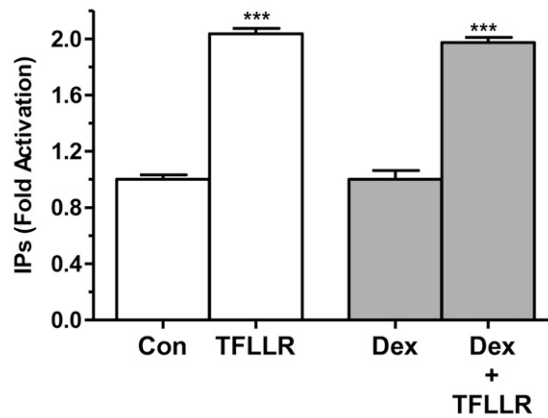

Fig. S8. PAR1 stimulation of inositol phosphates is unaffected by a 1-h cotreatment with glucocorticoids. A549 cells were treated for 1 h with vehicle (Con, control), 60 μM TFLLR, 100 nM Dex, or both 60 μM TFLLR and 100 nM Dex applied together, and total [^{3}H]inositol phosphates (IPs) were measured. Data were normalized to total lipids and plotted as fold increase over basal levels. Data represent mean ± SD from three experiments. ***$P < 0.001$.

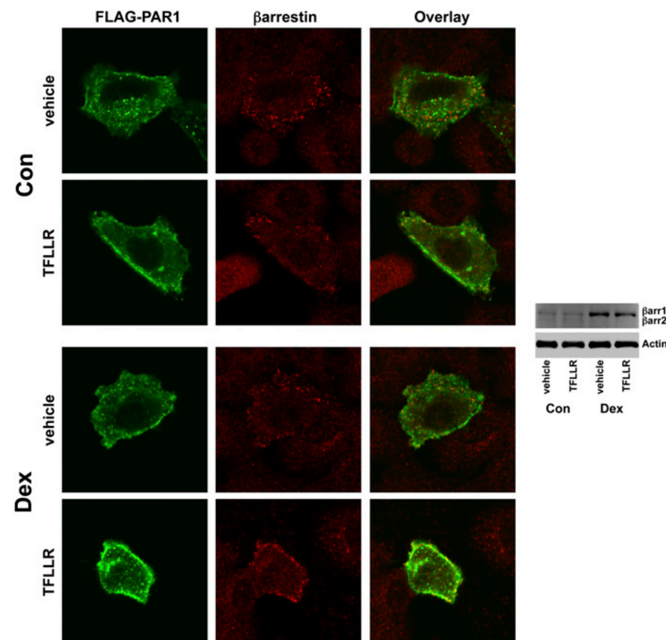

Fig. S9. Colocalization of β-arrestin with TFLLR-activated FLAG-PAR1 at the plasma membrane of A549 cells. A549 cells transiently expressing FLAG-PAR1 were pretreated with vehicle (Con, control) or 100 nM Dex for ~42 h. The cells were then stimulated with or without the PAR1-activating peptide TFLLR (100 μM) for 5 min, fixed, and processed for immunocytochemistry. Shown are representative confocal images of FLAG-PAR1 immunofluorescence (green) and β-arrestin immunofluorescence (red). Colocalization of β-arrestin with the receptor at the plasma membrane is indicated by yellow on the overlay. (*Right*) Immunoblots of β-arrestin-1 and β-arrestin-2 from cells processed in parallel.