The β2-adrenergic receptor (∝β2AR) has provided a paradigm to elucidate how G protein–coupled receptors (GPCRs) control intracellular signaling, including the discovery that β-arrestins, which bind to ligand-activated GPCRs, are central for GPCR function. We used genome editing, conditional gene deletion, and small interfering RNAs (siRNAs) to determine the roles of β-arrestin 1 (∝β-ar1) and ∝β-ar2 in β2AR internalization, trafficking, and signaling to ERK. We found that only ∝β-ar2 was essential for β2AR internalization. Unexpectedly, β-ar1 and ∝β-ar2 and receptor internalization were dispensable for ERK activation. Instead, β2AR signaled through Gαs and Gβγ subunits through a pathway that involved the tyrosine kinase SRC, the adaptor protein SHC, the guanine nucleotide exchange factor SOS, the small GTPase RAS, and the kinases RAF and MEK, which led to ERK activation. These findings provide a molecular framework for β2AR signaling through β-arrestin–independent pathways in key physiological functions and under pathological conditions.

INTRODUCTION
G protein (heterotrimeric guanine nucleotide–binding protein)–coupled receptors (GPCRs) represent the largest family of cell surface proteins involved in signal transmission. These receptors play key roles in physiological processes, and their dysfunction contributes to some of the most prevalent human diseases (1). Hence, GPCRs are the direct or indirect target of more than 25% of drugs on the market (1, 2). The study of β2-adrenergic receptors (∝β2ARs) has provided a paradigm to elucidate the fundamental mechanisms by which GPCRs control intracellular signaling in key physiological functions and under pathological conditions (1, 3–7).

Upon ligand binding, β2AR undergoes a rapid conformational change, leading to its association with the Gα subunit of the heterotrimeric G protein Gαs (Gαs) and the consequent release of guanosine diphosphate bound to Gαs and its exchange for guanosine triphosphate (GTP) (3, 6, 7). Whereas GTP-Gαs initiates signal transmission resulting in 3′,5″-cyclic adenosine monophosphate (cAMP) accumulation, G protein–coupled receptor kinases (GRKs) phosphorylate the C terminus of β2AR (5). This leads to recruitment of β-arrestins, which associate with β2ARs and cause receptor desensitization by uncoupling receptors from G proteins and promoting their internalization through clathrin-coated pits (4, 5, 8, 9), resulting in G protein signal termination at the plasma membrane. In addition to desensitization of G protein signaling, β-arrestins have also been proposed to initiate their own signals that stimulate the mitogen-activated protein kinases (MAPks) extracellular signal–regulated kinase 1/2 (ERK1/2) (collectively referred herein as ERK) (8, 9). However, the respective roles of β-arrestin 1 (∝β-ar1) and ∝β-ar2 (also known as arrestin-2 and arrestin-3, respectively) in receptor internalization and ERK activation have not been fully elucidated (10), and the relative contribution of β-arrestins and Gαs to overall ERK activation by β2AR has been debated (9, 11, 12). This has hindered a thorough mechanistic understanding of how β2AR activates ERK and hence limited the full potential of interfering with GPCR signaling to ERK for therapeutic intervention in multiple diseases.

RESULTS
β-ar2, but not ∝β-ar1, is essential for β2AR internalization
To begin investigating the relative contributions of β-ar1 and ∝β-ar2 to receptor internalization and signaling and the potential for redundancy, we established flow cytometry assays to determine the cell surface amounts of FLAG-β2AR upon activation by its agonist, isoproterenol. Internalization of stably expressed native β2AR in human embryonic kidney (HEK) 293 cells occurred rapidly, reaching a maximum within 15 to 30 min (Fig. 1, A to C). A phosphorylation-deficient mutant FLAG-β2AR 3S, in which the C-terminal GRK phosphorylation sites were mutated to impair β-arrestin–induced desensitization and internalization (13), showed minimal isoproterenol-stimulated receptor internalization, thus serving as a control (Fig. 1, A to C). Similarly, stimulation of cells at 4°C completely abolished receptor internalization (Fig. 1, B and C). As an additional control, the constitutive uptake of fluorescently labeled transferrin was monitored in HEK293 FLAG-β2AR and FLAG-β2AR 3S cell lines, which showed no significant differences (fig. S1A). To evaluate β-arrestin function in β2AR internalization, we performed small interfering RNA (siRNA) knockdown in HEK293 FLAG-β2AR cells (Fig. 1D). Knockdown of ∝β-ar1 alone had little effect on receptor internalization compared with siRNA control (siCont) (Fig. 1E). However, knockdown of β-ar2 alone largely impaired β2AR internalization, similar to knockdown of both ∝β-ar1 and ∝β-ar2, suggesting an important role for ∝β-ar2.
Fig. 1. Internalization of β₂AR in HEK293, β-arrestin KO, and β-arrestin–less cells. (A) Schematic of FLAG-β₂AR and FLAG-β₂AR 3S internalization assays. Iso, isoproterenol. (B) Surface FLAG-β₂AR or FLAG-β₂AR 3S abundance in HEK293 cells before or after 15 min of isoproterenol (10 μM) stimulation compared with isotype-stained reference control and control stimulation at 4°C. (C) Internalization of FLAG-β₂AR or FLAG-β₂AR 3S after isoproterenol stimulation; mean of three independent experiments as in (B) ± SD. (D) Western blot of β-arrestin 1 and 2, and α-tubulin (loading control) in HEK293 cells transfected with β-arrestin or control siRNAs. Representative of four independent experiments. (E) Internalization of FLAG-β₂AR after isoproterenol stimulation in HEK293 cells transfected with β-arrestin or control siRNAs; mean of three independent experiments ± SD. (F) Flow cytometry quantification of median fluorescence intensity (MFI) of 15-min transferrin-546 uptake in HEK293 FLAG-β₂AR cells transfected with control siRNA (siCont) or indicated siRNAs; mean of three independent experiments ± SD. ns, not significant. (G) Schematic of the β-arrestin TALEN construct design targeting exon 6. (H and I) Western blot for β-arrestins in (H) HEK293 and β-arrestin KO cells and in (I) HEK293 and β-arrestin KO cells transfected with two different siRNAs (A, B) targeting β-arrestin 1 or 2 or siRNA pools (A+B) or control scrambled siRNA (C). (J) Internalization of FLAG-β₂AR after isoproterenol stimulation in indicated cells; mean of three independent experiments ± SD.

but not β-arrestin 1, in β₂AR internalization (Fig. 1E). Transferrin uptake was similar under all conditions (Fig. 1F).

Advances in genome editing strategies with the development of TALEN (transcription activator–like effector nucleases) and CRISPR (clustered regularly interspaced short palindromic repeats) techniques have now made it feasible to investigate the effects of precise gene deletion (14, 15), as opposed to relying entirely on transient siRNA knockdown approaches. We used TALEN genome editing targeting β-arrestin 1 to generate a β-arrestin 1 knockout (KO) HEK293 cell line (Fig. 1, G and H, and fig. S1B). An antibody that detects both β-arrestin 1 and 2 did not yield a signal in the β-arrestin KO line, which agreed with previous reports that β-arrestin 1 is generally more highly abundant (16, 17). Nevertheless, the use of a β-arrestin 2–specific antibody indicated the presence of β-arrestin 2 in both the parental HEK293 and β-arrestin 1 KO lines (Fig. 1H). Two independent siRNAs targeting each β-arrestin effectively knocked down their respective target proteins, and the siRNAs were pooled for further experiments that compared the effects of β-arrestin 1 or 2. The β-arrestin 1 KO cells transfected with the pool of siRNAs targeting β-arrestin 2 were henceforth designated β-arrestin–less cells (Fig. 1I). Similar to the siRNA knockdown approach, the HEK293 β-arrestin 1 KO cells expressing FLAG-β₂AR had similar β₂AR internalization patterns as the HEK293 FLAG-β₂AR control cells (Fig. 1J). However, the loss of β-arrestin 2 in the β-arrestin–less cells greatly impaired β₂AR internalization (Fig. 1J) without affecting transferrin uptake (Fig. 1C). β-arrestin 2 is required for β₂AR trafficking to clathrin-coated pits and internalization into endocytic vesicles

We next took advantage of the use of the SNAP-tag system to specifically label cell surface–expressed β₂AR to visualize the internalization process by live cell confocal imaging and immunofluorescence of fixed cells (Fig. 2A). Aligning with our observations by flow cytometry internalization analysis using FLAG-β₂AR cells, HEK293 and β-arrestin 1 KO cells readily internalized SNAP-labeled β₂AR into early endosomes upon stimulation with isoproterenol (Fig. 2B and movies S1 and S2). However, SNAP-β₂AR predominantly remained...
We verified by total internal reflection fluorescence (TIRF) microscopy that FLAG-β2AR colocalized with EEA1 in early endosomes (Fig. 2B and movie S3). Immunofluorescence of SNAP-β2AR internalization into endocytic vesicles through clathrin-coated pits. Representative of three independent experiments.

**β-arrestins are dispensable for ERK activation**

In addition to receptor internalization, β-arrestins are proposed to desensitize β2AR signaling while concomitantly initiating β-arrestin–dependent signaling to ERK (9, 11, 18). Strikingly, the β-arrestin–less cells did not show decreased ERK phosphorylation but instead showed increased ERK activation in response to β2AR stimulation when compared with control HEK293 cells, using both In-Cell Western (Fig. 3A) and traditional Western blot (Fig. 3B) approaches. Although these results were unexpected, ERK activation was similarly increased rather than decreased in cells expressing the FLAG-β2AR 3S mutant that had impaired internalization and β-arrestin recruitment (Figs. 1, A to C, and 3, C to E). These data collectively suggest that initiation of ERK signaling from β2AR does not require receptor internalization or β-arrestins.

The enhanced signaling from β2AR to ERK in β-arrestin–less cells was confirmed by the increased sensitivity to the synthetic (isoproterenol) and natural (epinephrine) β2AR agonists by Western blot and phosphorylated ERK enzyme-linked immunosorbent assay (ELISA) analysis of dose-response curves. This approach revealed higher maximal responses ($E_{\text{max}}$) and lower median effective concentration (EC$_{50}$) values in β-arrestin–less cells compared with control cells (Fig. 3F). We also investigated the effects of β-arrestin depletion on cAMP signaling. Both β-arrestin knockdown and β-arrestin KO cells had increased cAMP production in

---

**Fig. 2. β2AR internalization into endocytic vesicles through clathrin-coated pits.** (A) Schematic of SNAP-tagged β2AR used for immunofluorescence (IF) imaging. Immunofluorescence of SNAP-β2AR and EEA1 endocytic vesicles (B) and respective Pearson’s correlation (R) values (C). Mean of 12 individual cells imaged from two independent experiments ± SEM in the indicated cells upon 10-min isoproterenol stimulation. Statistical significance was determined by t test. Scale bars, 10 μm. (D) Surface FLAG-β2AR abundance in the indicated cells after isoproterenol stimulation, determined by TIRF microscopy using fluorescently labeled β-arrestin2 siRNA. Representative of three independent experiments. (E) TIRF imaging of FLAG-β2AR (green) and clathrin-coated pits (red) in indicated cells before and after 5-min isoproterenol stimulation. Graphs depict the overlap in fluorescence intensity between FLAG-β2AR and clathrin-coated pits across designated lines through the cells, as an indication of colocalization. Scale bars, 5 μm; inset scale bars, 500 nm. Representative of three independent experiments.

---

on the cell surface in β-arrestin–less cells (Fig. 2B and movie S3). Immunofluorescence analysis with the early endosome marker EEA1 revealed that SNAP-β2AR colocalized with EEA1 in early endosomes in isoproterenol-stimulated control cells and β-arrestin1 KO cells, but not in β-arrestin–less cells, as quantified by Pearson’s coefficient analysis (Fig. 2, B and C).

We verified by total internal reflection fluorescence (TIRF) microscopy that FLAG-β2AR endocytosis was abolished in β-arrestin–less cells (Fig. 2D), whereas β-arrestin1 KO cells maintained similar amounts of internalization compared with control HEK293 cells, in agreement with the flow cytometry and immunofluorescence data. We then examined β2AR accumulation in clathrin-coated pits, the key step in the process of ligand-induced endocytosis of β2AR that is promoted by β-arrestins. β2AR clustered rapidly in clathrin-coated pits after isoproterenol application in HEK293 and β-arrestin1 KO cells, whereas receptor clustering in clathrin-coated pits was abolished in β-arrestin–less cells (Fig. 2E, fig. S2A, and movies S4 to S6). Additionally, knockdown of clathrin heavy chain disrupted FLAG-β2AR internalization, verifying that β2AR internalization was clathrin-dependent in these cells (fig. S2B). Thus, our data obtained through multiple complementary approaches indicate that β2AR internalization occurs predominantly through clustering of receptors into clathrin-coated pits that is specifically β-arrestin–dependent.
response to isoproterenol that was greater in the β-arrestin-less cells (fig. S3). These data support the idea that β-arrestins restrain β2AR-initiated ERK activation and cAMP accumulation. Moreover, we also used the CRISPR/Cas9 system to genetically delete both β-arrestin 1 and β-arrestin 2 simultaneously (Fig. 3G and fig. S4, A to D). Similar to the β-arrestin-less cells, two separate β-arrestin 1/2 double-KO clonal cells showed stronger phosphorylation of ERK at early time points compared with the parental control cells (Fig. 3H). This complementary genome editing approach provided further support to the notion that β-arrestins are dispensable for ERK activation by β2AR. The amounts of endogenous and transfected β2AR were similar between parental HEK293 and β-arrestin 1/2 KO cell lines, as measured by specific binding of [3H]dihydroalpenrolol ([3H]DHAP), suggesting that differences in receptor amounts did not account for the differences in signaling observed (fig. S3A). Furthermore, we verified that the β-arrestin 1/2 double-KO lines had abolished β2AR and arginine vasopressin receptor 2 (V2R) internalization upon stimulation with their respective ligands, isoproterenol and arginine vasopressin (AVP) (figs. S5, B and C). These observations were consistent with an important role for β-arrestin 2 in receptor-mediated internalization (Figs. 1 and 2). We also observed an increase in isoproterenol-mediated ERK activation in β-arrestin 1/2 double-KO lines relative to the parental HEK293 cells with endogenous receptor amounts (Fig. 3I), suggesting that receptor overexpression was not affecting the results. In addition, we compared the effects of the loss of β-arrestin 1/2 on ERK activation by another GPCR, V2R. Similar to results observed with β2AR, β-arrestin 1/2 was dispensable for V2R-mediated ERK activation (fig. S6).

ERK activation by β2AR is Gαs-dependent

β2AR is predominantly coupled to Gαs. Thus, in the search for the underlying mechanism(s) linking β2AR to ERK, we tested whether Gαs coupling was required for isoproterenol-mediated ERK phosphorylation. Using mouse embryonic fibroblasts (MEFs) derived from Gnas−/− mice, we generated a stable FLAG-β2AR-expressing cell line (fig. S7A). Cells infected with control adenovirus exhibited a robust activation of ERK, whereas excision of Gαs using a Cre–green fluorescent protein (GFP) adenosivirus abrogated isoproterenol-induced ERK phosphorylation (Fig. 4A). Thus, conditional Gαs gene deletion eliminated ERK activation in MEFs. As a complementary approach, we used Gαs-deleted HEK293 cells lacking both of the expressed Gαs gene members (GNAS and GNAL), generated using the CRISPR/Cas9 system (Fig. 4B and fig. S7B). Compared with the parental cells, the GNAS KO HEK293 cells had nearly complete impairment of ERK activation by isoproterenol, whereas control epidermal growth factor (EGF)–mediated activation of ERK was preserved (Fig. 4C). Disruption of Gαs signaling in these cells was confirmed by cAMP and CRE luciferase assays and by its restoration by Gαs reexpression (Fig. 4, D and E). Similarly, transfection of Gαs into GNAS KO HEK293 cells effectively restored robust ERK signaling (Fig. 4F), supporting the notion that Gαs coupling is critical for initiating β2AR-mediated ERK activation.

PKA is dispensable for ERK activation by β2AR

Because protein kinase A (PKA) is a major signaling target of Gαs and cAMP production, we next tested the role of PKA in β2AR-mediated ERK activation. Similar to previously published results (11), we observed that pretreatment with the PKA inhibitor H89 impaired ERK activation by isoproterenol (Fig. 4G). However, H89 is not a very specific inhibitor and can target multiple additional kinases (19, 20). Thus, we tested more selective inhibitors of PKA including the cell-permeable competitive antagonist cAMPS-RP. In contrast to H89, cAMPS-RP had little effect on isoproterenol-induced ERK activation (Fig. 4H), although it effectively blocked isoproterenol- and forskolin-induced CRE-dependent transcription (Fig. 4I). As a complementary approach, we expressed a GFP-tagged version of protein kinase A inhibitor (PKI), a specific peptide inhibitor of PKA, or control PKI mutant peptide (PKI-Mut), in which the PKA inhibitory domain is disrupted (21). The PKI peptide, but not the control PKI-Mut peptide or GFP plasmid alone, disrupted CRE luciferase induction by isoproterenol stimulation (Fig. 4J). However, as with cAMPS-RP, inhibition of PKA with PKI did not impair isoproterenol-induced ERK phosphorylation (Fig. 4J). Because PKA is not the only target of cAMP production downstream of Gαs, activation, we also tested the potential role of EPAC, a RAPI guanine nucleotide exchange factor, in transmitting signals, leading to ERK activation. However, CE3F4, a small molecule that blocks EPAC-induced RAPIA activation, also failed to reduce isoproterenol-induced ERK activation (fig. S7C), although it successfully disrupted RAPIA activation (fig. S7D).

ERK activation by β2AR is mediated by SRC, SHC, SOS, and RAS

Similar to the absence of direct evidence that PKA is required for β2AR-mediated ERK activation, pharmacological agents inhibiting EGF receptor (EGFR) (AG1478), phosphatidylinositol 3-kinase (PI3K) (LY294002), and Gαs (pertussis toxin) also failed to prevent ERK activation in response to isoproterenol in HEK293 FLAG-β2AR cells (fig. S8, A to C). Thus, we next decided to delineate signaling upstream of ERK activation after the classic RAS/RAF/MAPK kinase (MEK)/ERK signaling cascade, although, based on our data, it was unlikely that RAF activation could be achieved by a β-arrestin–dependent activation bypassing RAS, as previously proposed (22, 23). The MEK1/2 inhibitor U0126 completely abrogated ERK phosphorylation in response to isoproterenol and EGFR (the latter was used as a control), indicating that MEK1/2 was strictly required (Fig. 5A). Working upstream, the B-RAF inhibitors SB-590885 and GDC-0879 similarly abrogated ERK phosphorylation in response to isoproterenol and EGFR (Fig. 5B). To evaluate the role of RAS in the activation of ERK, we used dominant-negative KRAS and HRAS plasminids. Transfection of either dominant-negative RAS plasmin reduced the isoproterenol-induced ERK activation, with the KRAS dominant-negative plasmin having the more potent inhibitory effect (Fig. 5C). Although expression of dominant negative KRAS substantially disrupted β2AR-mediated ERK phosphorylation, it did not prevent the induction of CRE luciferase by isoproterenol or forskolin, supporting a role for RAS in ERK activation independently of cAMP/PKA-induced Cre transcription (Fig. 5D). Pull-down assays for active Ras indicated that active GTP-bound Ras was increased upon isoproterenol stimulation (Fig. 5E). In a complementary approach, we generated stable FLAG-β2AR–expressing MEFs in which Hras and Nras genes were deleted and the Kras gene was floxed (fig. S8D) (24). Stimulation of these “Rasless MEFs,” in which treatment with Cre–GFP adenosivirus efficiently excised the Kras gene, substantially reduced ERK phosphorylation in comparison to adeno-GFP control (Fig. 5F), collectively supporting a key role for RAS in ERK activation by β2AR.

To explore the role of candidate RAS-GEFs involved in activating the RAS/RAF/MEK/ERK signaling cascade, we performed a RAS guanine nucleotide exchange factor (GEF) siRNA library screen, which included siRNAs for each of the eight members of the RAS-GEF superfamily (25) and the In-Cell Western blot fluorescence...
Fig. 3. ERK activation in HEK293 and β-arrestin−less cells. (A) In-Cell Western blot with quantification of ERK phosphorylation (pERK) induced by isoproterenol stimulation, performed in triplicate ± SEM; representative of two independent experiments shown. (B) Western blot and quantification of isoproterenol-induced pERK normalized to total ERK from three independent Western blots ± SEM. Statistical significance was determined by t test. Westerns are from the same blot and exposure; representative of three independent experiments. (C) Immunofluorescence of FLAG-β2AR and FLAG-β2AR 3S in HEK293 cells upon 5-min isoproterenol stimulation. Scale bars, 10 μm. Representative image from two independent experiments. (D) Western blot of pERK and indicated controls upon isoproterenol stimulation of HEK293 FLAG-β2AR or FLAG-β2AR 3S cells. (E) Quantification of pERK normalized to ERK from three independent Western blots ± SEM in the indicated cells after isoproterenol stimulation. Statistical significance was determined by t test. (F) pERK Western blots of the indicated cells after 3-min isoproterenol and epinephrine (Epi) stimulations. (G) Western blot for pERK, total ERK, and α-Tubulin on April 24, 2018 http://stke.sciencemag.org/ Downloaded from

Cont but not PKA, is critical for β2AR-mediated ERK phosphorylation. (A) Immunofluorescence showing GFP expression of FLAG-β2AR Gnas f/f MEFs transduced with control adenoviral (adeno)-GFP or adenov-Cre-GFP and Western blot of pERK upon 3-min isoproterenol stimulation of these cells (representative of three independent experiments). (B) Western blot for Gαs in HEK293 CRISPR/Cas9-edited Gαs KO cells. (C) Western blot of pERK upon 3-min isoproterenol or EGF (10 ng/ml) stimulation in the indicated cells. Representative of three independent experiments. (D and E) Relative amounts (mean ± SEM of three independent experiments) of cAMP (D) and CRE luciferase activity (E) in the indicated cells upon isoproterenol stimulation. (F) Western blot of pERK after isoproterenol stimulation in Gαs KO FLAG-β2AR cells transfected with vector control or Gαs. Representative of three independent experiments. (G and H) Western blot of pERK in HEK293 FLAG-β2AR cells stimulated with isoproterenol after pretreatment with dimethyl sulfoxide (DMSO) (control), H89 (10 μM) (G), or cAMPS-RP (100 μM) (H). Representative of three independent experiments for (G) and (H). (I) Relative CRE luciferase activity in isoproterenol- and forskolin (FSK; 5 mg/ml)-stimulated HEK293 FLAG-β2AR cells pretreated with H89, cAMPS-RP, or DMSO control; mean ± SEM of three experiments. (J) Immunofluorescence of GFP expression, relative CRE luciferase activity (mean ± SEM, three experiments), and Western blot of pERK in HEK293 FLAG-β2AR cells transfected with GFP-PKI or GFP-PKI-mutant (PKI-Mut) plasmids upon stimulation with isoproterenol (representative of three independent experiments).

 assay described above. Among the GEFs tested, SOS1 and SOS2 had the most significant z scores, therefore representing the best candidates for mediating isoproterenol-induced ERK phosphorylation in β2AR-expressing HEK293 cells (Fig. 5G). We confirmed a critical role for SOS1 and SOS2 in β2AR-mediated ERK activation by Western blot analysis of cells transfected with a pool of siRNAs targeting SOS1 and SOS2 (Fig. 5H). Because SHC and upstream SRC family kinases can activate SOS-RAS signaling, we investigated whether β2AR stimulation mediated phosphorylation of SRC and SHC. Isoproterenol stimulation caused an increase in tyrosine phosphorylation of SHC and SRC (Y416) (Fig. 5I). Additionally, the small-molecule SRC inhibitors SU6656 and PP1 impaired the isoproterenol-induced phosphorylation of SRC and SHC (Fig. 5J) and substantially reduced ERK activation (Fig. 5K). Cells depleted of β-arrestins (β-ar--less cells) showed largely normal isoproterenol-induced phosphorylation of SRC (Fig. 5L). Therefore, SRC appears to play an important role in β2AR-mediated ERK activation that is independent of β-arrestins. Finally, we examined whether Gβγ signaling upon Gαs activation may be important for ERK activation. An HA-tagged C-terminal GRK2 construct (HA-GRK2ct), which selectively blocks βγ signaling (also known as βARKct) (26), substantially reduced the β2AR-mediated ERK phosphorylation without affecting EGF-mediated ERK activation (Fig. 5M). Additionally, inhibition of βγ signaling through GRK2ct also significantly disrupted the phosphorylation of SRC and SHC (Fig. 5N and fig. S8E). Thus, Gβγ signaling appears to play a key role in the initiation of a signaling cascade involving SRC, SHC, SOS, RAS, RAF, MEK, and ERK that links β2AR to ERK (Fig. 6).

**DISCUSSION**

The scaffolding and proposed activation of ERK by β-arrestins has received considerable attention due to the critical role of ERK in cell growth, survival, and proliferation responses. Additionally, ERK activation is frequently monitored in high-throughput drug screens for “biased” GPCR agonists and antagonists, in which G
protein signaling may be inhibited, whereas β-arrestin function is maintained, and vice versa. Although β-arrestins have been proposed to interact with and induce their own G protein–independent activation of ERK (9, 27), our results showed that loss of β-arrestins enhanced the potency and efficacy of isoproterenol- and epinephrine-induced ERK phosphorylation. Therefore, it is possible that the effects of β-arrestins on desensitization of G protein signaling, including G protein–dependent ERK activation, override their potential direct contributions to ERK activation. Hence, not only cAMP signaling but also ERK phosphorylation responses are enhanced in β-arrestin–depleted and KO cells.

Overall, the combination of genome editing approaches with siRNA-mediated knockdowns, conditional gene deletion in engineered fibroblasts, and genome editing cell KOs for critical signaling nodes provided genetic evidence supporting that β-arrestins play distinct roles in β2AR internalization and desensitization, including a preferential role for β-ar2 in β2AR internalization (4, 5, 8, 28), while revealing that β-arrestins are dispensable for ERK activation and
function. Specifically, our findings are consistent with a model in which β2AR stimulation promotes the activation of Gaαs, promoting the accumulation of cAMP through Gaαs and ERK activation primarily through Gβγ subunits (Fig. 6). In the absence of Gβγ signaling, the limited residual ERK activation may suggest the existence of additional minor contributing mechanisms that may require further elucidation, such as a cAMP-dependent activation that is not strictly necessary for β2AR activation of ERK (Fig. 6). In turn, Gβγ may stimulate the kinase activity of SRC directly, as has been previously demonstrated using in vitro reconstitution systems and purified components (30, 31), and the sequential activation of Ras and the ERK kinase cascade through SOS1/2 upon SHC phosphorylation by SRC (Fig. 6). Overall, our results support that initiation of ERK activation by β2AR involves a signaling route that is independent of β-arrestins, with β-arrestins inhibiting rather than activating ERK in this pathway.

These findings may have general implications for signaling to ERK through Gaαs-coupled GPCRs. Specifically, β2AR is a prototypical GPCR that associates transiently with β-arrestins, often referred to as class A GPCR, as compared to class B GPCRs, which include AT1A angiotensin receptors and V2R vasopressin receptors and associate tightly with β-arrestins and are co-internalized (31). The use of β-arrestin KO cells suggests that, at least for V2R, β-arrestins are not strictly required for ERK signaling by this class B GPCR. ERK phosphorylation downstream of activation of the free fatty acid receptor 4 (FFA4), which couples with Gaαs, depends on Gaαs but not β-arrestins (32). Thus, the dispensable role of β-arrestin in GPCR-induced ERK activation is likely to be generalizable and is supported by several additional studies (33, 34). On the other hand, the role of receptor internalization for prolonged ERK activation by class B GPCRs has been traditionally interpreted as β-arrestin-initiated ERK signaling (9). However, class B GPCRs form a ternary complex, with β-arrestins binding to the C-terminal phosphorylated tail of the GPCR and Gaαs remaining bound to the receptor, likely locked in a signaling-competent conformation (35). This observation may help explain the fact that many GPCRs can promote long-lasting accumulation of cAMP from an intracellular compartment, including early endosomes (36), through Gaαs that is co-internalized with the GPCRs and β-arrestins (37). Thus, knockdown reduces ERK activation by the β2AR (39), opposite to its effect on ERK activation by β2AR. Thus, it is possible that functional diversity between receptors is achieved through receptor-specific differences in the degree to which ERK activation is limited by β-arrestins’ distinct signal-attenuating and scaffolding functions. Cell type-specific differences in signaling specificity may also come into play, and so, future studies using new genome editing approaches may help clarify these issues in specific cell populations.

The use of genome editing strategies and targeted gene deletion in MEFs provides enormous power for improving knowledge of GPCR and β-arrestin function by the genetic dissection of receptor-specific differences in signaling, desensitization, and trafficking. Overall, we could link β-arrestins to desensitization of β2AR-mediated signaling and identify a selective function for β-arrestin2 in endocytosis of β2AR mediated by clathrin-coated pits. Analysis of ERK activation also revealed the need for caution when using ERK as a screen for β-arrestin-biased agonists and for considering β-arrestins in overall ERK signaling and function because we observed a critical role for Gaαs signaling and a desensitizing role of β-arrestins in the overall ERK activation. Thus, the use of genome-edited cellular systems to screen for biased drug responsiveness may provide better insights into the mechanisms by which they exert their pharmacological effects. Overall, we have defined a molecular framework by which β2AR initiates ERK activation by a β-arrestin-independent pathway. Ultimately, our findings provide a mechanism by which multiple GPCRs may control ERK-regulated gene transcription programs and the consequent orchestration of cell-specific biological responses that are dependent on ERK.

MATERIALS AND METHODS
Reagents
Isoproterenol, forskolin, EGF, epinephrine, thrombin, U0126, PP1, SU6656, and H89 were purchased from Sigma. 8-(4-Chlorophenylthio)adenosine-3′,5′-cyclic monophosphate (8-CPT-cAMP) and 8-(4-chlorophenylthio)-2′-O-methyl-cAMP (8-pCPT-2′-O-Me-cAMP) were from BIOLOG Life Science Institute (Axonora). CE3F4, cAMPS-RP, SB-590885, and GDC-0879 were obtained from Tocris. Pertussis
toxin (PTX) was obtained from List Biological Laboratories Inc. AG1478 and LY294002 were obtained from Calbiochem. siRNAs targeting β-arr1, β-arr2, clathrin heavy chain (CHC-17), and AllStars Negative Control were purchased from Qiagen. Sandwich ELISA kit for phospho-ERK1/2 and antibodies against phospho-ERK1/2 [phospho-p44/p42 MAPK (Thr202/Tyr204) XP], ERK1/2 (p44/42 MAPK), β-arr1, β-arr2, β-ar1/2, phospho-Akt (Ser473) (D9E) XP, Akt, phospho-SRC (Tyr416), SRC (32G6), phospho-SHC (Tyr239/240), and α-tubulin (DM1A) were from Cell Signaling Technology. Gαs, Gαo, S011, and S022 antibodies were obtained from Santa Cruz Biotechnology. SHC antibody was from Millipore/Upstate Biotechnology. Pan-RAS (Ab-3) antibody was from Calbiochem. Antibodies against HA and Myc (9E10) tags were obtained from Covance. FLAG–phycocerythrin (PE) antibody (P315) was from Prozyme. EEA1 and clathrin heavy chain antibodies were from Santa Cruz Biotechnology. Alexa Fluor 488 and 546 labels were purchased from New England Biolabs. Puromycin, LI-COR imaging were from Invitrogen. SNAP surface Alexa Fluor 488 was from CellLight. SNAP–2AR was expressed in pcDNA3.1 vector for transient transfection. 3×FLAG–GRK2ct was prepared performing polymerase chain reaction (PCR) on GRK2 amino acid residues 495 to 689, with the addition of an N-terminal 3×HA tag, and inserted into our in-house pCEFL vector. The resulting construct was sequenced with the Sanger sequencing method (Fasmac). 3×HA–AVP2R DNA was purchased from cDNA Resource Center (Bloomburg University, Bloomburg, PA). The HA-tagged GRK2ct was prepared performing polymerase chain reaction (PCR) on GRK2 amino acid residues 495 to 689, with the addition of an N-terminal HA tag, and inserted into our in-house pCEFL vector. Construct sequence was verified by DNA sequence analysis (National Institute of Dental and Craniofacial Research shared resource facility). Human GNAS open reading frame (short isoform) was PCR-amplified from reverse-transcribed human total mRNA (FirstChoice Human Total RNA Survey Panel, Ambion) and cloned into a pCAGGS expression vector (a gift from J. Miyazaki, Osaka University, Japan). The inserted fragment was sequenced with the Sanger sequencing method (Fasmac).

**Cell culture**

HEK293, Rasless MEFs, and Gnas l/f MEFs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and penicillin/streptomycin (Sigma). Rasless MEFs were derived from Nr2s and Hras KO mice harboring a floxed allele of Kras as previously described (24). Gnas l/f MEFs were provided by J. Regard and generated as previously described (41). Gαo, KO HEK293 cells were prepared using CRISPR/Cas9 as previously reported (42).

**Generation of the β-arr1 TALEN KO HEK293 cell line**

The Cornell TALEN Targeter software was used to identify β-arr1 TALEN sites and check for potential off-target sites (https://tale-nt.cac.cornell.edu/). The β-arr1 TALEN was designed to target the following sequence corresponding to a region of exon 6, in which capital letters correspond to TALEN binding sites and the lowercase letters correspond to the spacer region sequence: TCCCCTCAAAACCTTCCATGTTCACGACGCGGGAA. TALENs were assembled using the EZ-TAL Assembly Kit (System Biosciences), and the repeat-variable diresidue (RVD) sequences of the assembled β-arr1 TALENs were as follows: TAL1 RVD, HD HD NG HD HD NI NI HD HD NG NG HD NI NG NG; TAL2 RVD, NG HD HD HD HD NH NG NG HD NG HD HD NH NIH HD HD. A surveyor nuclease assay was performed to confirm targeting of the TALEN at the appropriate β-arr1 locus. Briefly, genomic DNA was isolated using the QiAamp DNA Isolation Kit (Qiagen) and amplified with the AccuPrime SuperMix (Life Technologies) using oligos surrounding the TALEN cut site (forward primer: GTTCAAGAAAGGCCGTCACAAATTGGAAMGC; reverse primer: CTGATGGGTTCCTCCTCAATGTAATAGATCTCC). A surveyor nuclease assay was then performed on PCR-amplified DNA from control- or TALEN-transfected HEK293 cells using the Surveyor Mutation Detection Kit (Transgenetic Inc.). To generate the β-arr1 TALEN KO (β-arr1 KO) cell line, HEK293 cells were transfected using Turbofect transfection reagent (Fermentas) and clonally selected by limited dilution. Single-cell clones were expanded and then screened by surveyor nuclease assay and Western blot for loss of β-arr1.
vector (0.25 μg) and the ARR22-targeting vector (0.25 μg) was transfected into the cells using 1.25 μL of Lipofectamine 2000 (Life Technologies). After a 3-day incubation, the cells were harvested with trypsin/EDTA, and GFP-positive cells were isolated using an SH800 cell sorter (Sony). The GFP-positive cells were diluted with DMEM supplemented with 10% FBS and penicillin/streptomycin and seeded in 96-well plates to isolate single clones using a limiting dilution method. The seeded 96-well plates were incubated for about 2 weeks with routine addition of fresh medium and observed under microscope for their colony appearances. While passaging clonal cells into a six-well plate, an aliquot of cell suspensions was analyzed for mutations in the targeted sites using PCR and restriction enzyme digestion. For mutational analysis of the ARR21 target #1, PCR was performed with primers 5′-TTAGATGGGACATGCTTGCG-3′ and 5′-GAGTGGTCTCTGAGAGATCT-3′, and the PCR amplicon was digested with Apa I (Takara Bio). Similarly, the following primers and restriction enzymes were used: for the ARR21 target #2, primers 5′-GGATCCTCCTCCGAGTTTCTC-3′ and 5′-CTACGCTCTGGTACGATGATC-3′ and Hap II; for the ARR22 target #1, primers 5′-ACGTTAGGTATATGGACGTCC-3′ and 5′-TACGCGTCTGAGGTTGATC-3′ and Nco I; for the ARR22 target #2, primers 5′-TCTGCGGCTTACGTCATCC-3′ and 5′-CCCCAGGGTCTAATTCACTGC-3′ and Xho I. A PCR program started with an initial denaturation cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 15 s, 64°C for 30 s, and 72°C for 30 s. The resulting PCR product (5 μL) was digested with a corresponding restriction enzyme (0.5 μL) in a reaction buffer (total volume of 10 μL) and incubated at 37°C for 1 h. Digested DNA fragments were analyzed with the MultiNA Microchip Electrophoresis System (Shimadzu). Candidate clones that harbored restriction enzyme–resistant PCR fragments were further assessed for their genomic DNA alterations by TA cloning. Lack of functional β-arrestins was also confirmed by assessing protein expression and ligand-stimulated GPCR internalization as described below. For the TA cloning, PCR-amplified genomic DNA fragments using an Ex Taq polymerase (Takara Bio) were gel-purified (Promega) and cloned into a pMD20 T-vector (Takara Bio). Ligated products were introduced into SCS1 competent cells (Stratagene), and transformed cells were selected on an ampicillin-containing LB plate. At least 12 colonies were picked, and inserted vectors were PCR-amplified using the Ex Taq polymerase and primers [5′-CAGGAACAGCTATGAC-3′ (M13 Primer RV) and 5′-GTTCGCTCTTCGAGCACAC-3′ (M13 Primer MR)] designed to anneal the pME20 T-vectors. PCR products of the transformed pMD20 T-vector were sequenced using the Sanger method (Fasmac) and the M13 Primer RV.

**Transfection, RNA interference, and viral transduction**

Transient transfections of plasmid DNAs were performed using Lipofectamine 2000 (Invitrogen) reagent for 48 hours. Transfections of siRNAs were performed using Lipofectamine RNAiMAX (Invitrogen) using 50 nM siRNAs for 72 hours to achieve RNA interference–mediated knockdown. Cells transfected with both siRNA and plasmid DNA were first transfected with siRNA for 24 hours, followed by a medium change before transfection with plasmid DNA for another 48 hours. FLAG-β2AR and FLAG-β2AR 35 lentiviruses were prepared by transfecting HEK293T/17 cells (ATCC CRL-11268) with pL8EP FLAG-β2AR, psPAX2, and vesicular stomatitis virus glycoprotein in a 3:2:1 ratio using TurboFect transfection reagent (Fermentas) and collected 48- and 72-hour viral supernatants. HEK293, β-arr1 KO cells, Rasless MEFs, and Gnas Δf MEFs were transduced by infection with 0.45 μM polynosinedil fluoride (PVDF)–filtered FLAG-β2AR lentivirus with polybrene (6 μg/ml) and then selected with puromycin (1 μg/ml) (Invitrogen) to generate stable lines. Expression of FLAG-β2AR was verified by flow cytometry using a FLAG–PE–conjugated antibody (Prozyme) and analyzed on a FACSCalibur (BD Biosciences). Adenoviral transductions of Rasless MEFs and Gnas Δf MEFs with adenov-PE control or adenov-PE Icre to delete Kras or Gnas (G0), respectively, involved infection of cells with 2.8 × 107 plaque-forming units/ml of virus plus polybrene (6 μg/ml) for 72 hours. Infection was confirmed by fluorescence microscopy for GFP expression and Western blot analysis for loss of Ras or G0 in the Cre-infected MEFs.

**Internalization assays**

Internalization assays were performed by FLAG-PE surface staining of stably transduced FLAG-β2AR HEK293 and β-arr1 KO cells before and after specified time points of stimulation with 10 μM isoproterenol. Cells were incubated on ice and quickly rinsed with ice-cold phosphate-buffered saline (PBS). Cells were lifted with 1 mM EDTA in PBS, centrifuged at 3000 rpm for 5 min at 4°C, and stained in a 100-μl volume of 0.5% bovine serum albumin (BSA)–PBS with 1.25 μl of FLAG-PE (Prozyme) antibody covered on ice. Cells were washed three times with 1 ml of cold 0.5% BSA-PBS and then analyzed on a FACSCalibur (BD Biosciences). Transferrin internalization assays were performed by incubating cells in serum-free DMEM with or without conjugated transferrin–Alexa Fluor 546 (Invitrogen) for 15 min followed by washes with DMEM and PBS and resuspension in 0.5% BSA-PBS for flow cytometry. Flow cytometry data analysis and MRI values were calculated using FlowJo analysis software on live-gated cells. Percent internalization was calculated based on MRI values as follows: % internalization = (1 – MRI time point/MRI unstimulated) × 100.

**Cre luciferase assay**

Cre luciferase assays were performed by seeding HEK293 stably transduced with FLAG-β2AR cells in a poly-lysine–coated 24-well plate and culturing in DMEM supplemented with 10% FBS for 24 hours. Cells were then cotransfected with 100 ng of plasmid DNA or control, 50 ng of Cre-firefly luciferase reporter DNA, and 20 ng of pRL-Renilla luciferase using Lipofectamine 2000 (Life Technologies) transfection reagent. The day after transfection, cells were serum-starved, treated with appropriate inhibitors when relevant, and then stimulated for 6 hours with isoproterenol (10 μM) before harvesting the cells. For all Cre-luciferase assays, cells were lysed and luciferase activity was determined using the Dual-Glo Luciferase Assay Kit (Promega). Chemiluminescence was measured using a BioTek Synergy Neo plate reader, and Cre activation was calculated as the ratio of firefly to Renilla luciferase levels. The assays were performed three times in duplicate.

**Confocal microscopy**

Confocal immunofluorescence and live cell images were collected on a Zeiss LSM 700 laser scanning microscope with a 40× oil immersion lens in a multitrack mode using dual excitation (488 nm for Alexa Fluor 488 and 555 nm for mRFP) and emission (band-pass, 505 to 515 nm for Alexa Fluor 488; long-pass, 560 nm for mRFP) filter sets. For experiments examining SNAP-β2AR trafficking, HEK293 and β-arr1 KO cells transfected with SNAP-β2AR and siRNAs as specified were seeded in poly-lysine–coated 35-mm glass-bottomed culture dishes for live cell (MatTek Corporation) or 1-mm coated glass...
coverslips (Fisher) in a six-well plate for fixed immunofluorescence imaging. Cells were labeled with appropriate SNAP surface Alexa Fluor dyes for 30 min at 37°C and then washed three times with complete medium before imaging. Cells for immunofluorescence imaging were stimulated for indicated times with isoproterenol and then washed with PBS and fixed with 2% formaldehyde/PBS solution for 12 min. Fixed cells were blocked in 10% FBS/PBS and then permeabilized with 0.05% saponin (Sigma) solution and incubated with EEA1 (BD Biosciences) antibody for 1 hour at room temperature. Coverslips were washed three times for 5 min with 10% FBS/PBS and then stained with anti-mouse Alexa Fluor 546 antibody for 1 hour at room temperature. Coverslips were washed again and then mounted onto glass slides (Fisher) with FluorSave (Calbiochem) mounting solution.

Live-cell TIRF microscopy imaging
TIRF microscopy was performed at 37°C using a Nikon Ti-E inverted microscope equipped for through-the-objective TIRF microscopy and outfitted with a temperature-, humidity-, and CO2-controlled chamber (Okolab). Images were obtained with an Apo TIRF 100×, 1.49–numerical aperture objective (Nikon) with solid-state lasers of 488, 561, and 647 nm (Keysight Technologies) as light sources. An IXon DU+ DU-897 Andor camera controlled by NIS-Elements 4.1 software was used to acquire image sequences every 2 s for 10 min. HEK293 cells were transfected with the appropriate Alexa Fluor 647–labeled siRNAs for 24 hours, followed by a medium change before transfection with FLAG-β2AR and clathrin light chain–dsRed for 48 hours. Cells were plated on poly-L-lysine (Sigma)–coated 35-mm glass-bottomed culture dishes (MatTek Corporation). Before imaging, cells were labeled 1:1000 with M1 FLAG antibody conjugated to Alexa Fluor 488 dye for 10 min at 37°C, washed, and imaged live in DMEM without phenol red [University of California, San Francisco (UCSF) Cell Culture Facility] supplemented with 30 mM Hepes (pH 7.4) (UCSF Cell Culture Facility). Cells were stained with bath application of 10 μM isoproterenol at frame 10 of 301 image sequences.

TIRF microscopy image analysis
Acquired image sequences were saved as stacks of 16-bit TIFF files. All quantitative image analysis was performed on unprocessed images using ImageJ software (http://rsb.info.nih.gov/ij, U.S. National Institutes of Health). To quantify change in receptor fluorescence over time, fluorescence values of individual cells were measured in five randomly selected regions of interest (ROIs) in the cell over the entire stack. An area of the coverslip-lacking cells was used to estimate background fluorescence. Fluorescence values of the five ROIs were background-subtracted, averaged, normalized to initial fluorescence values before agonist addition, and then normalized to photobleaching control images. Minimal bleed-through and photobleaching was verified using single-labeled and untreated samples, respectively. To quantify receptor concentration into clathrin-coated pits, a line selection was drawn through several clathrin light chain–dsRed foci, representing clathrin-coated pits, at the indicated time points. The plot profile function was used to measure pixel values along this line in the clathrin light chain–dsRed and FLAG-β2AR channels. Pixel values were then normalized and represented as a percent of maximum fluorescence for each channel.

Western blot and pERK ELISA
For cell stimulations for Western blot, HEK293 and β-ar1 KO cells transfected with siRNAs and Rasless MEFs and Gnas i/f cells treated for 72 hours with adeno-iCre or adeno-GFP control were left untreated or treated with 10 μM isoproterenol at indicated time points. Additional 3-min stimulations with forskolin (5 mg/ml) or EGF (10 ng/ml) were also performed when indicated. For the experiments done with pharmacological inhibitors, confluent cells were first treated with the vehicle control (DMSO), LY294002 (25 μM) for 1 hour, AG1478 (10 μM) for 1 hour, U0126 (10 μM) for 90 min, PP1 (10 μM) for 1 hour, SU6656 (10 μM) for 1 hour, H89 (10 μM) for 1 hour, C3P4 (10 μM) for 1 hour, cAMPS-RP (100 μM) for 30 min, SB-203580 (10 μM) for 4 hours, GDC-0879 (10 μM) for 4 hours, or PTX (50 ng/ml) for 16 hours before isoproterenol stimulation. For V2R stimulations, 100 nM AVP was used at the indicated time points. Cells were lysed in radioimmunoprecipitation assay buffer (Sigma) containing a mixture of protease and phosphatase inhibitors (Thermo Fisher Scientific) and clarified by centrifugation. Lysates were resolved on SDS–polyacrylamide gel electrophoresis gels, transferred onto PVDF membranes (Millipore), and probed with appropriate antibodies. Westerns to detect active RAS-GTP were performed using glutathione-Sepharose beads (GE Healthcare) immobilized with the RAS-interactive binding domain of c-Raf-1 fused to glutathione S-transferase as previously described (44). For pERK1/2 ELISA (Sandwich ELISA kit #7177, Cell Signaling Technology), cells were cultured in a 12-well plate, stimulated with indicated concentrations of ligands, and lysed in 150 μl of provided lysis buffer supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific). ELISA was performed according to the manufacturer’s instructions, and absorbance readings were measured on a BioTek Synergy Neo plate reader.

In-Cell Western blot and siRNA RAS-GEF library screen
Cells for In-Cell Western blot were cultured to near confluence in a 96-well plate and stimulated for indicated periods of time with 10 μM isoproterenol, then the medium was discarded, and cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature. Cells were then permeabilized with 0.5% Triton X-100 with 200 mM glycine in PBS for 10 min at room temperature. After washing with PBS, cells were blocked with 3% BSA-PBS for 1 hour at room temperature. The wells were then incubated in primary antibodies diluted in 3% BSA-PBS (rabbit phospho-ERK1/2 at 1:100 and mouse total ERK1/2 at 1:200) overnight at 4°C with gentle rocking. The wells were washed three times with 0.1% Tween 20 in PBS for 10 min at room temperature with gentle shaking. They were then incubated with secondary antibodies diluted in 3% BSA-PBS (LI-COR anti-mouse 680 at 1:1000 and anti-rabbit 800 at 1:1000) for 1 hour at room temperature with gentle rocking and covered with foil to protect from light. The wells were then washed three times with 0.1% Tween 20 in PBS for 10 min at room temperature with gentle shaking and then imaged on the Odyssey imager. The siRNA Ras-GEF library screen was performed using an In-Cell Western screen with knockdown of a library of Ras-GEFs [Life Technologies, D1337380 (custom gene group)] in HEK293 cells expressing FLAG-β2AR in the presence and absence of isoproterenol stimulation (10 μM).

cAMP assays
CAMP accumulation from endogenous β2ARs after isoproterenol stimulation (10 μM) was measured using plasmid pGLO-20F (Promega), which encodes a circularly permuted luciferase cAMP reporter. HEK293 cells were transfected with appropriate siRNAs for 24 hours, followed by a medium change before transfection with pGLO-20F plasmid DNA using Lipofectamine 3000 (Invitrogen) transfection reagent for 24 hours. Cells were then assayed as previously
described (37). For each siRNA treatment, reference wells were treated with 5 μM forskolin, and all experimental cAMP measurements were normalized to the maximum luminescence value measured in the presence of forskolin to control for cell number and DNA transfection efficiencies after siRNA treatment. Another cAMP assay was performed using the HTRF CAMP assay kit from CisBio (cAMP dynamic 2 kit #62AM4PEB) to monitor cAMP levels stimulated by isoproterenol (10 μM) in HEK293 control cells, Goα KO cells, and Goα KO cells reconstituted with Goαα. Fluorescence measurements were made using a Victor2 plate reader.

Membrane preparation

HEK293 parental and β-ar1/2 KO cell lines were transfected with FLAG-β2AR DNA, and membranes were harvested 48 hours later at confluency. Membranes were harvested in warm harvesting buffer [0.68 mM EDTA, 150 mM NaCl, and 20 mM Hepes (pH 7.4)] and washed with PBS. Cell suspension was centrifuged for 3 min at 2000 rpm, and the supernatant was removed. Cells were homogenized in cold membrane preparation buffer [10 mM Hepes, 10 mM NaCl, 0.5 mM MgCl2, and 0.5 mM EGTA (pH 7.4)] and centrifuged at 15,000 rpm for 20 min at 4°C. The process was repeated twice, and the supernatant was removed. Cells were resuspended in cold binding assay buffer [10 mM Hepes, 10 mM NaCl, 0.5 mM MgCl2, and 1 mM ascorbic acid (pH 7.4)] and sonicated. Protein concentrations were measured by DC protein assay (Bio-Rad).

Radioiodine binding assay

In a polypropylene 96-well plate, binding assay buffer or propionol (50 μM final for nonspecific binding), [3H]DHAP (2 nM final), and membranes (5 μg per well) were plated in order and incubated for 2 hours at room temperature to reach equilibrium. GF/C filterplates were prepared with 0.3% polyethyleneimine to minimize nonspecific binding. Samples were transferred to the filterplates, washed with cold assay buffer, and dried overnight. Each well was counted in MicroScint-0 (PerkinElmer) for 1 min in triplicate.

Statistical analyses

Statistical analyses of data were performed using GraphPad Prism 7 software (GraphPad Software). The data were analyzed by analysis of variance (ANOVA) test or t test. The mean differences of three independent experiments were considered significant when P values were <0.05. Nonlinear regression (least squares fit) analysis was used to fit curves and determine the EC50 and Emax values.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/cgi/content/full/10/484/eaal3395/DC1

Fig. S1. Transferin uptake experiments and genomic DNA sequencing of the β-ar1 KO cell line. Fig. S2. Clathrin is important for isoproterenol-mediated β-ar internalization. Fig. S3. Depletion of both β-ar1 and β-ar2 increases β-ar-mediated cAMP production. Fig. S4. Sequence of β-ar1/2 double-mutant cells. Fig. S5. Endogenous β-ar in parental HEK293 and β-ar1/2 KO and β-ar and V2R internalization in these cells. Fig. S6. β-Arrestin is not required for ERK phosphorylation in arginine vasopressin receptor 2 (V2R)-expressing cells. Fig. S7. Stable expression of FLAG-β-ar on Gnas IT/IT MEFS and Goα KO cells and effect of EPAC inhibition on isoproterenol-mediated ERK phosphorylation and RAP1A activation. Fig. S8. EGFR, PKC, and Goa signaling are dispensable for activation of ERK by β-ar, but Goβ signaling is important. Movie S1. Live-cell confocal imaging of β-ar internalization in HEK293 cells. Movie S2. Live-cell confocal imaging of β-ar internalization in β-ar1 KO cells. Movie S3. Live-cell confocal imaging of β-ar internalization in β-ar2-less cells. Movie S4. Live-cell TIRF imaging of β-ar internalization into clathrin-coated pits in HEK293 cells.


Acknowledgments: We thank M. Barbacid and E. Santos, and J. Regard for providing the Rasless (Hras and Nras KO and floxed Hras) and Gnas floxed MEFs used in these studies, respectively. pCAGGS expression vector was a gift from J-i Miyazaki (Osaka University). The pSpCas9(BB)-2A-GFP (PX458) vector was a gift from F. Zhang (Broad Institute). Funding: This study was partially supported by the National Institute of Dental and Craniofacial Research intramural program at the NIH (J.S.G. and M.O.). A.J. received funding from Japan Science and Technology Agency, Precursory Research for Embryonic Science and Technology (JPMJPR1331) and the PRIME, Japan Agency for Medical Research and Development, and J.A. received funding from Japan Agency for Medical Research and Development, Core Research for Evolutional Science and Technology. J.S.G. received funding from University of California, San Diego (UCSD) Moores Cancer Center and Department of Pharmacology. M.u.Z. received funding from the National Institute on Drug Abuse (DA012864 and DA06511). K.E. is a recipient of an NSF Graduate Research Fellowship. D.J.S. was supported in part by the UCSD Graduate Training Program in Cellular and Molecular Pharmacology through an institutional training grant from the National Institute of General Medical Sciences (T32 GM007752). Author contributions: M.O., K.E., and A.I. analyzed the data. M.O., K.E., A.I., J.A., M.v.Z., and J.S.G. performed experiments. M.O., K.E., S.A., X.Z., X.F., K.K., D.J.S., and A.I. performed the experiments. M.O., K.E., and A.I. analyzed the data. M.O., K.E., A.I., M.v.Z., and J.S.G. designed the experiments. M.O. and J.S.G. wrote the paper, and all authors reviewed and edited the paper. Competing interests: The authors declare that they have no competing interests.
Genetic evidence that β-arrestins are dispensable for the initiation of β₂-adrenergic receptor signaling to ERK

Morgan O’Hayre, Kelsie Eichel, Silvia Avino, Xuefeng Zhao, Dana J. Steffen, Xiaodong Feng, Kouki Kawakami, Junken Aoki, Karen Messer, Roger Sunahara, Asuka Inoue, Mark von Zastrow and J. Silvio Gutkind

Sci. Signal. 10 (484), eaal3395.
DOI: 10.1126/scisignal.aal3395

β-Arrestins not necessary

The β₂-adrenergic receptor is thought to activate signaling mediated by the kinase ERK through a pathway that does not require G proteins but rather the β-arrestin family of scaffolding proteins. Biased agonists for the β₂-adrenergic receptor are being developed to selectively activate this pathway. O’Hayre et al. took advantage of improved technologies for knocking out proteins in cells and unexpectedly found that β-arrestins were not required for ERK activation downstream of the β₂-adrenergic receptor, although β-arrestin 2 was required for receptor internalization. Instead, the pathway depended on G α₃, Gβγ, and various other signaling molecules. These results suggest that biased agonists for the β₂-adrenergic receptor may exert their effects in a β-arrestin–independent manner.