Science Signaling
AAAS

www.sciencesignaling.org/cgi/content/full/4/185/ra51/DC1

Supplementary Materials for

Distinct Phosphorylation Sites on the β_2-Adrenergic Receptor Establish a Barcode That Encodes Differential Functions of β-Arrestin

Kelly N. Nobles, Kunhong Xiao,* Seungkirl Ahn, Arun K. Shukla, Christopher M. Lam, Sudarshan Rajagopal, Ryan T. Strachan, Teng-Yi Huang, Erin A. Bressler, Makoto R. Hara, Sudha K. Shenoy, Steven P. Gygi, Robert J. Lefkowitz*

*To whom correspondence should be addressed. E-mail: lefko001@receptor-biol.duke.edu (R.J.L.); khxiao@receptor-biol.duke.edu (K.X.)

Published 9 August 2011, *Sci. Signal.* **4**, ra51 (2011)
DOI: 10.1126/scisignal.2001707

The PDF file includes:

Figure S1.

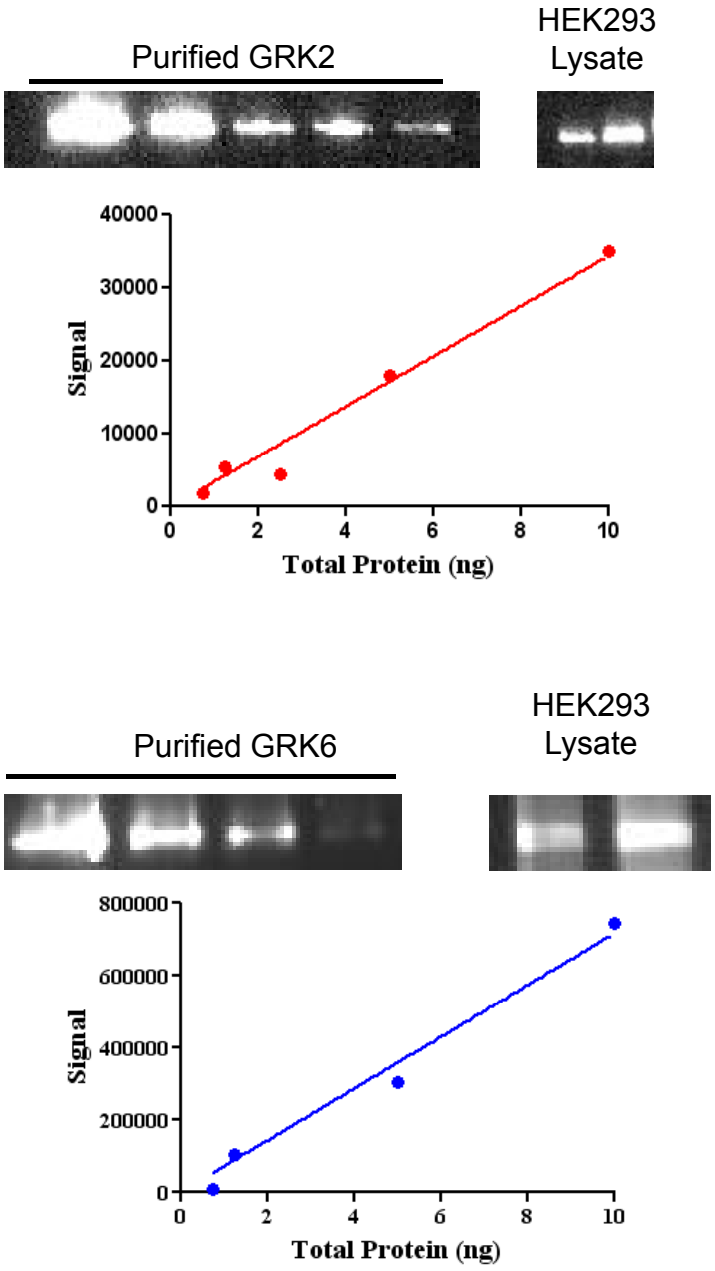


Figure S1. Endogenous GRK2 and 6 in HEK293 cells. HEK293 cell lysate was prepared using glycerol lysis buffer as described under Western blot analysis of Materials and Methods. Known amounts of purified GRK2 and GRK6 were analyzed by Western blot analysis with varying amounts of HEK293 lysate on the same nitrocellulose filters. Signals were quantified by densitometry and the data points for the known GRK amounts were analyzed with linear regression. Signals from the "unknowns," or HEK293 lysate, were then used to solve the equation of line to determine GRK2 and 6 protein amounts. The absolute amounts of GRK2 and GRK6 in HEK293 cells were determined as 4.5 ± 1.2 fmol/mg and 3.0 ± 0.6 fmol/mg, respectively.

Figure S2.

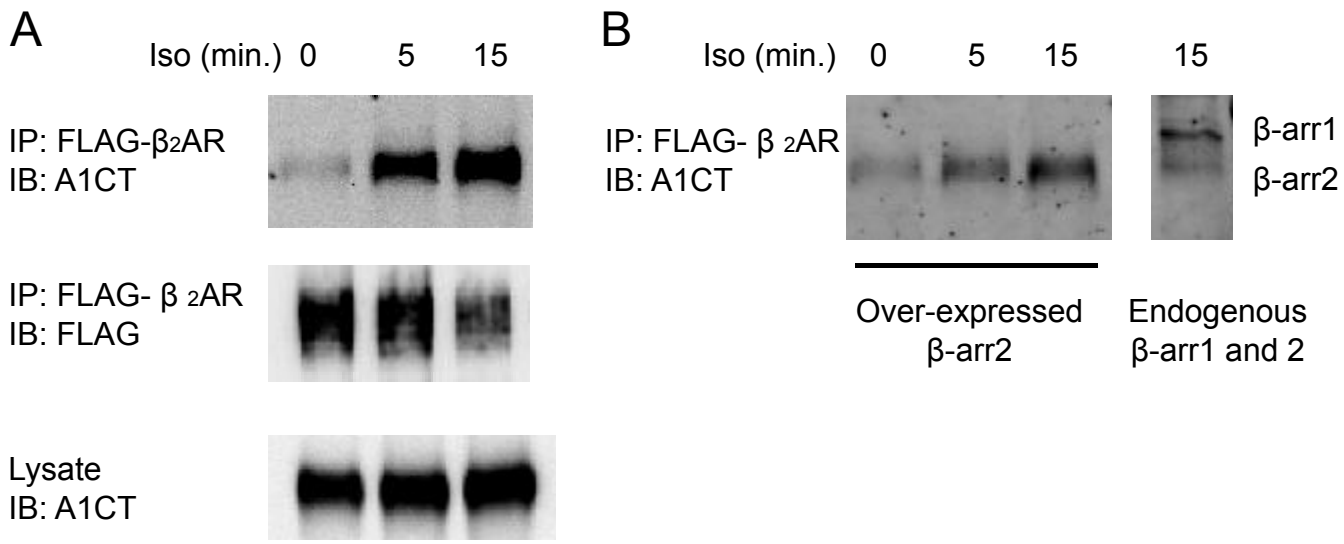


Figure S2. Specific β-arrestin2 recruitment to the β2AR. HEK293 cells stably expressing FLAG-β2AR were stimulated with isoproterenol for the indicated time points. (**A**) Western blot analyses were performed with dithiobis(succinimidyl)propionate cross-linked and FLAG immunoprecipitated (IP) β2AR from cells transiently transfected with β-arrestin2. The β-arrestin2 and FLAG-β2AR in the IPs were visualized by immunoblotting (IB) with A1CT (top panel) and FLAG (middle panel) antibodies, respectively. β-arrestin2 over-expression was confirmed with A1CT IBs using cell lysate (bottom panel). (**B**) Samples from (A) were diluted ten-fold in order to compare with endogenous β-arrestin1 and 2 (right panel) immunoprecipitated from this cell line.

Figure S3.

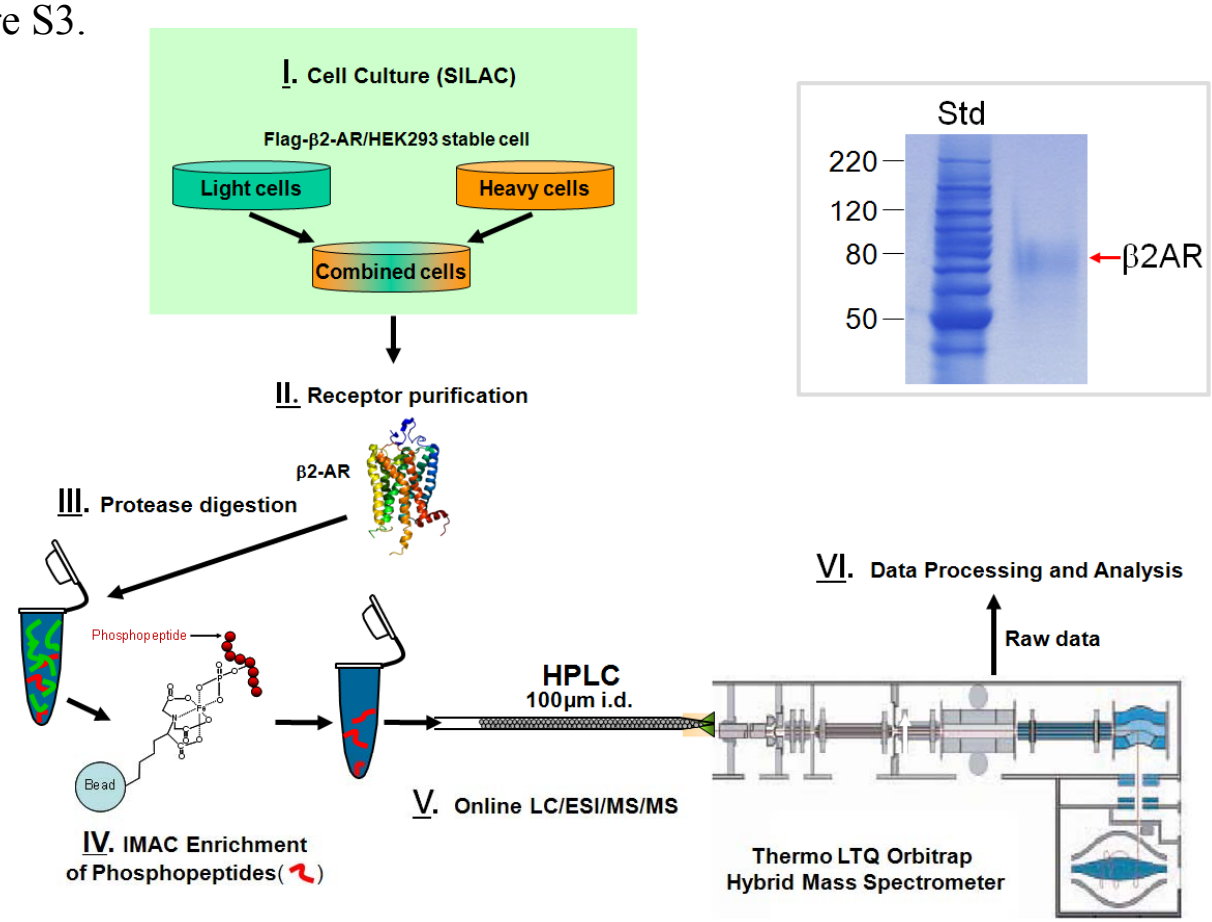


Figure S3. Overview of β_2AR phosphorylation analysis by MS-based quantitative proteomics. (Step I) HEK293 cells stably transfected with human β_2AR were used to prepare receptor samples for MS-based proteomic study in combination with a stable isotope labeling with amino acids in cell culture (SILAC) strategy. For quantitative analysis of the extents of phosphorylation of each site before and after agonist stimulation, "light" labeled cells were treated with agonist (10 μM isoproterenol or carvedilol) for 5 minutes, and "heavy" labeled cells served as controls without agonist treatment. Equal numbers of "heavy" and "light" cells were mixed at 1:1 ratio before receptor purification. For quantitative analysis of the extents of phosphorylation of each site when individual GRKs were depleted from cells, "heavy" cells were treated with GRK2 or 6 siRNA and "light" cells were treated with control siRNA. Both "heavy" and "light" cells were treated with 10 μM isoproterenol for 5 minutes and mixed at 1:1 ratio. (Step II) The β_2ARs were purified from the mixed "heavy" and "light" cells using an alprenolol-agarose affinity purification procedure. (Step III) The Purified β_2ARs were in-solution digested by protease. (Step IV) Phosphopeptides were enriched by IMAC (Immobilized Metal Ion Affinity Chromatography) and (Step V and VI) were analyzed by online liquid chromatography/tandem mass spectrometry (LC/MS/MS). Details are described in the main text and the Material and Method section. The insert shows a representative Coomassie stained SDS-PAGE gel of the purified β_2ARs. The position of the receptor was indicated and the molecular weights (kDa) of the protein standard (Std) were labeled.

Figure S4.

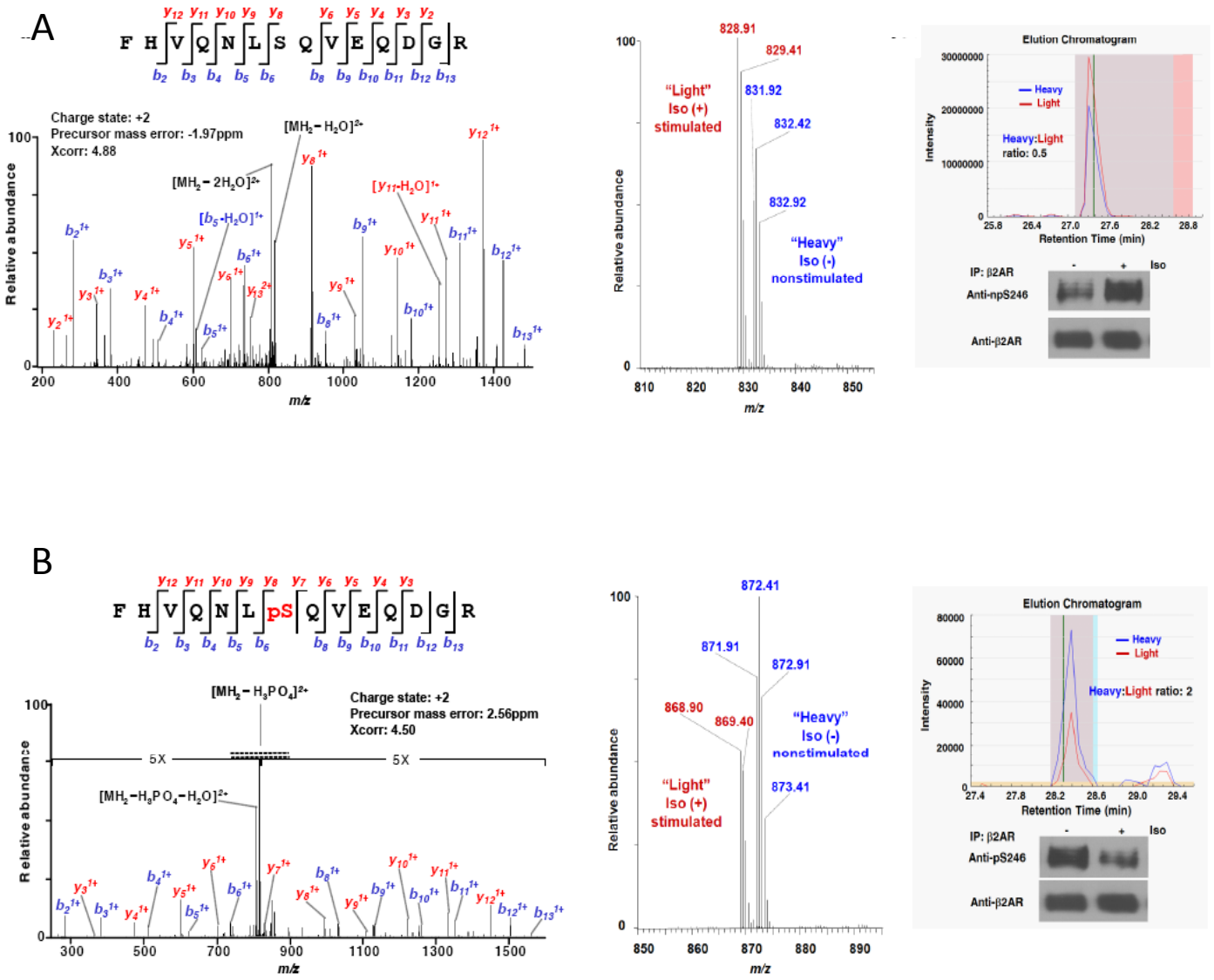
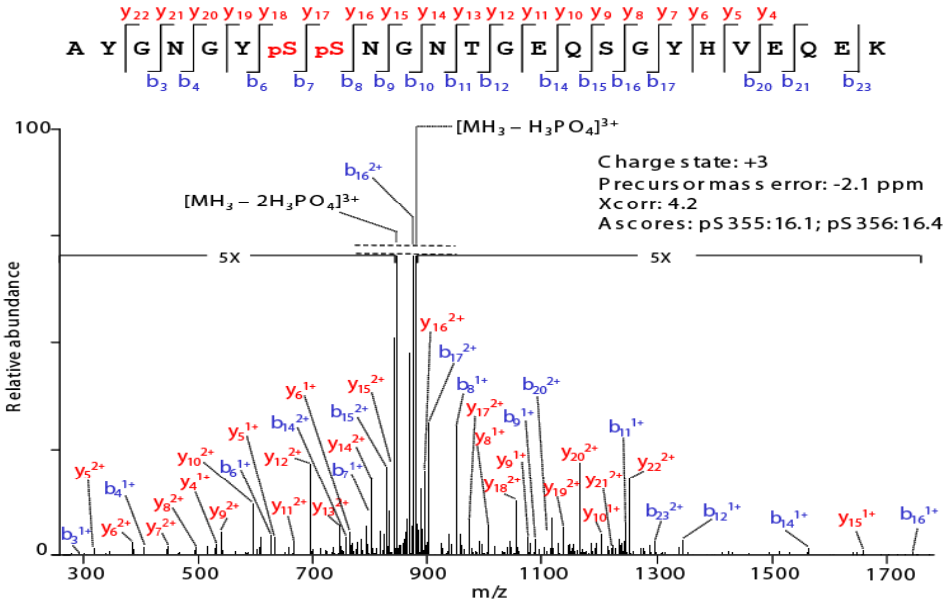

A
F H V Q N L S Q V E Q D G R
Charge state: +2
Precursor mass error: -1.97ppm
Xcorr: 4.88
Relative abundance
m/z
828.91
829.41
831.92
832.42
832.92
"Light"
Iso (+)
stimulated
"Heavy"
Iso (-)
nonstimulated
Elution Chromatogram
Heavy
Light
Heavy:Light
ratio: 0.5
Retention Time (min)
IP: β2AR
Iso
Anti-npS246
Anti-β2AR
B
F H V Q N L pS Q V E Q D G R
Charge state: +2
Precursor mass error: 2.56ppm
Xcorr: 4.50
5X
5X
872.41
871.91
872.91
868.90
869.40
873.41
"Heavy"
Iso (-)
nonstimulated
"Light"
Iso (+)
stimulated
Heavy:Light ratio: 2

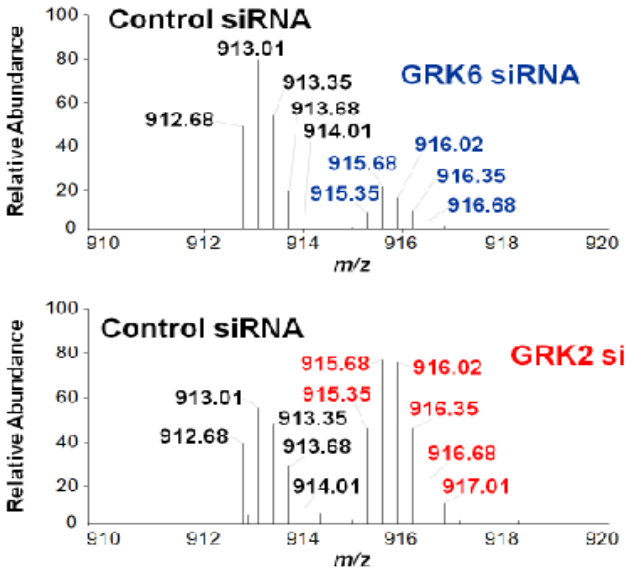
Figure S4. Dephosphorylation of β_2AR Ser^{246} by isoproterenol stimulation.
Phosphorylation of Ser^{246} on β_2AR was used as an example to demonstrate how the quantitative analyses were performed. (**A**) shows the quantification procedure for a tryptic non-phosphopeptide FHVQNLSQVEQDGR of β_2AR and the lower panels (**B**) shows that for the corresponding phosphopeptide FHVQNLpSQVEQDGR. The left panels present the annotated MS/MS fragmentation spectra for the "light" versions of the nonphosphopeptide and phosphopeptide, respectively. The peptide sequences are shown at the top of the MS/MS spectra with phosphorylated residues highlighted in red. The identified fragmentation *y* (red color) and *b* (blue color) ions are indicated. The middle panels are the expanded MS spectrum sections showing two isotope envelopes (peaks) corresponding to "light" and "heavy" forms of the unfragmented nonphosphopeptide and phosphopeptide, respectively. The "light" peaks (labeled with red color) represent the peptide from the agonist stimulated sample, whereas "heavy" peaks (labeled with blue color) represent those from the nonstimulated sample. The right panels show the extracted ion chromatograms for the nonphosphopeptide and phosphopeptide, respectively. The AUC (Area Under the Curve) was used for calculating the relative abundance of the "light" and "heavy" forms of a phosphopeptide. H/L, heavy to light ratio. Below the extracted ion chromatograms are Western-blot analyses using polyclonal antibodies generated with the same corresponding peptides. Anti-pSer^{246} is a phospho-specific antibody for β2AR-Ser^{246} and anti-npSer^{246} is a the antibody that recognize the corresponding site when it is not phosphorylated. Iso, isoproterenol; Iso (-), no treatment; Iso(+),isoproterenol treatment.

Figure S5.

A



B



C

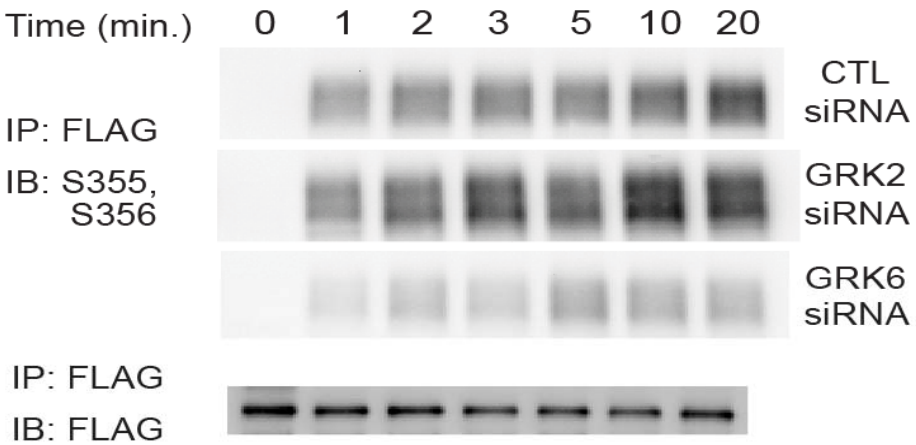


Figure S5. Phosphorylation of Ser^{355}/Ser^{356} on β_2AR as detected by LC-MS/MS and Western blotting. (**A**) Annotated MS/MS fragmentation spectra for the "light" phosphopeptide AYGNGYpSpSNGNTGEQSGYHVEQEK from the sample which was treated with control siRNA. The peptide sequence is shown at the top of the MS/MS spectrum with phosphorylated residues highlighted in red. The identified fragmentation *y* (red color) and *b* (blue color) ions are indicated. (**B**) is the expanded MS spectrum section showing two isotope envelopes (peaks) corresponding to "light" and "heavy" forms of the unfragmented phosphopeptides. The "light" peak (black color) represents the peptide from the control siRNA-treated sample, whereas "heavy" peak represents those from the GRK6 (blue color, upper panel) or GRK2 (red color, lower panel) siRNA-treated sample. The relative abundance of the "heavy" and "light" forms of a phosphopeptide was calculated by the ratio of AUC (Area Under the Curve) for "heavy" to "light" peaks in the extracted ion chromatogram. In this case there is an 80% reduction in phosphorylation of Ser^{355}/Ser^{356} when GRK6 is depleted. When GRK2 is depleted, the phosphorylation of Ser^{355}/Ser^{356} increased 1.5-fold. (**C**) The top three panels are Western blot analyses with a phospho-specific antibody recognizing pS^{355}/pS^{356} on β_2AR immunoprecipitated from stably transfected HEK-293 cells (2pmols/mg) that have been transfected with either control (CTL), GRK2 or GRK6 siRNA, respectively. The bottom panel is a FLAG Western blot to show equal loading of immunoprecipitated β_2AR.

Figure S6.

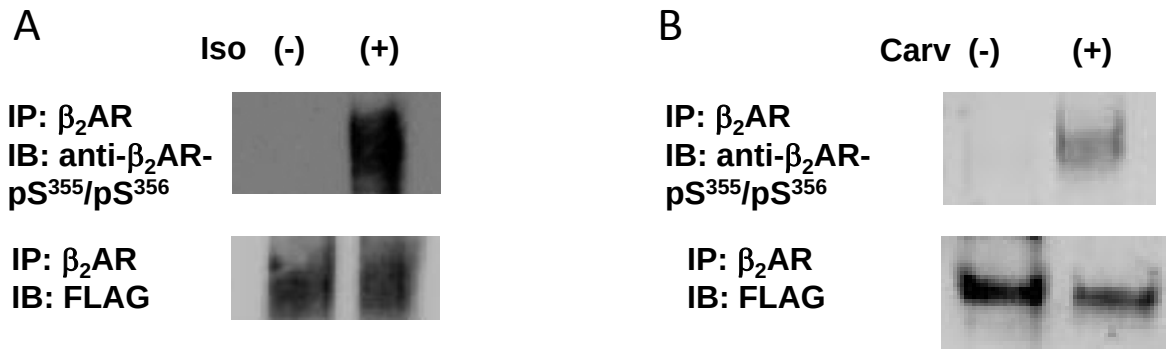


Figure S6. Western blot analysis of phosphorylation of β_2AR on Ser^{355}/Ser^{356} in response to isoproterenol and carvedilol stimulation. Phosphorylation of Ser^{355}/Ser^{356} on β_2AR was examined using a commercially available site-specific phospho-antibody (anti- β_2AR-pS^{355}/pS^{356}; Santa Cruz). β_2ARs were purified from HEK293 cells stably transfected with FLAG-tagged β_2AR using an alprenolol-agarose affinity purification procedure. The amount of the phosphorylation of β_2AR at Ser^{355}/Ser^{356} was measured by probing the receptor with the phosphorylation site-specific antibody. The bottom panel is a FLAG Western blot to show equal loading of the immunoprecipitated β_2AR. (**A**) The cells were treated with or without 10 μM isoproterenol for 5 minutes and then analyzed. (**B**) The cells were treated with or without 10 μM carvedilol for 5 minutes and then analyzed.

Figure S7.

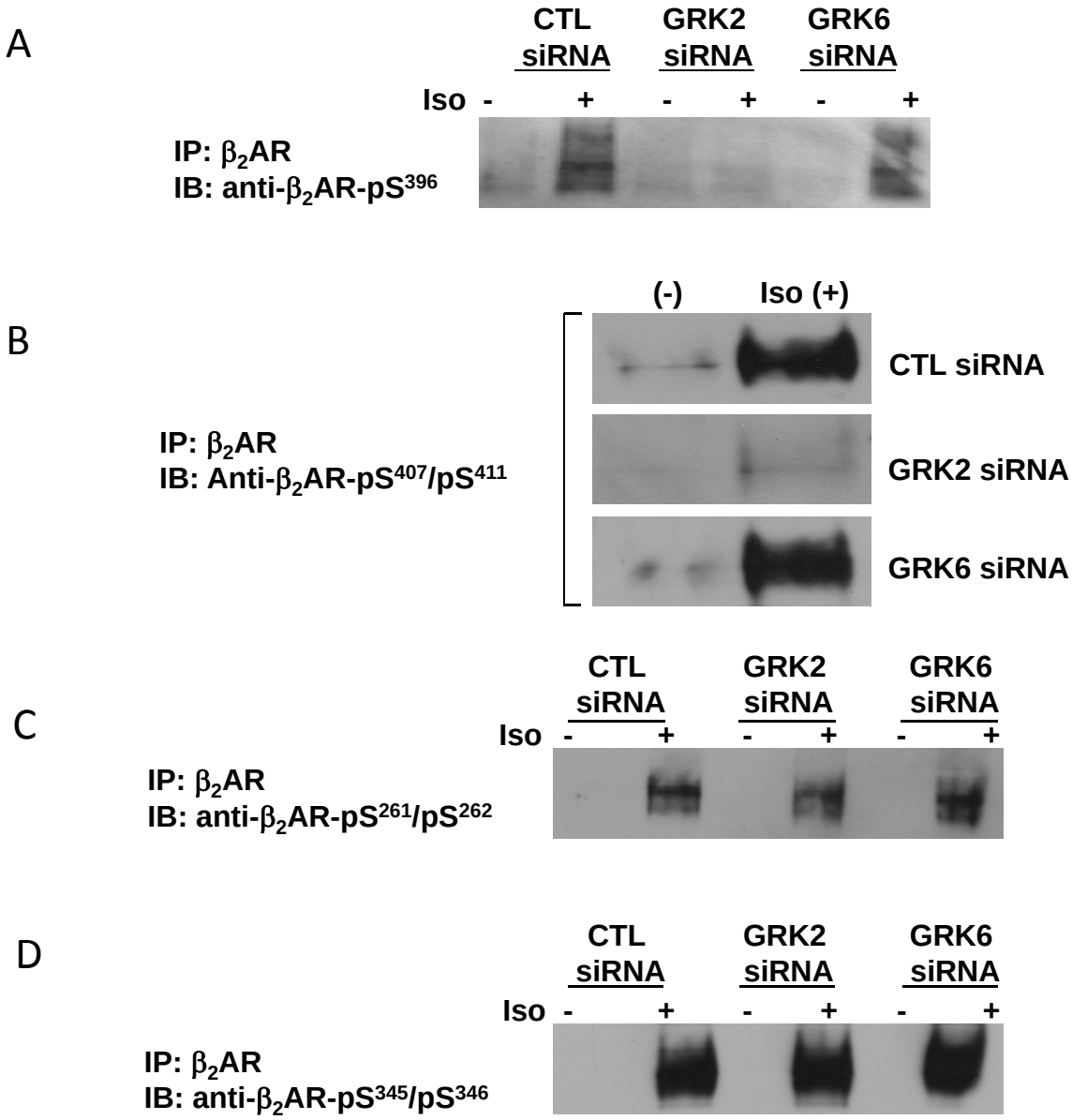


Figure S7. Western blot analysis of β_2AR phosphorylation at Ser^{396}, Ser^{407}/Ser^{411}, Ser^{261}/Ser^{262}, and Ser^{345}/Ser^{346}. β_2ARs were purified from the HEK-293 cells stably transfected with the β_2AR that have been transfected with either control (CTL-), GRK2- or GRK6- siRNA. Cells were stimulated with or without 10 μM isoproterenol for 5 minutes before the receptors were pulled down using an alprenolol-agarose affinity purification procedure. Western blot analysis was performed with the indicated phosphorylation-site specific antibodies. (**A**) β_2AR phosphorylation at Ser^{396}. (**B**) β_2AR phosphorylation at Ser^{407}/Ser^{411}. (**C**) β_2AR phosphorylation at the PKA-mediated phosphorylation sites Ser^{261}/Ser^{261}. (**D**) β2AR phosphorylation at the PKA-mediated phosphorylation sites Ser^{345}/Ser^{346}. Quantitative analyses of at least three independent experiments for each phosphorylation site-specific antibody are shown in Figure 5 of the main text.