Supporting Information

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SI Text

Cell Culture. HEK293 cells were maintained as previously described (1). The cells were transfected with cDNA encoding 2 μg FLAG-EGFR or EGFR-GFP with FuGENE reagent (Roche). Transfected cells were incubated overnight in serum-free medium supplemented with 0.1% BSA, 10 mM Hepes (pH 7.4), and 1% penicillin before stimulation. Under serum starvation condition, cells were preincubated with ICI-118551 and either PP2, GM 6001, HB-EGF neutralizing antibody, or AG1478 for 30 min, followed by stimulation with agonists or β-blockers for 5 min (immunoblotting) or 20 to 30 min (confocal microscopy) as described previously (2). Cell lines stably expressing WTβ_1AR, PKA$^-$ β_1AR, and GRK$^-$ β_1AR ($\approx$1 pmol/mg protein) have been previously described (3).

Immunoblotting and Detection. Following stimulation, cells were washed once with PBS solution, solubilized in 1 ml of lysis buffer (5 mM Hepes, 250 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 2 mM EDTA, and protease inhibitors) as previously described (1). Samples were resolved by SDS/PAGE and transferred to nitrocellulose membrane or PVDF (BioRad) for immunoblotting. Anti-phospho-EGFR (Tyr-845; Cell Signaling), phospho-ERK1/2 (Cell Signaling), and phospho-Akt (Cell Signaling) were used at 1:3,000. Immunoblotting for total EGFR (Upstate), total ERK (Upstate), and total AKT (Cell Signaling) was carried out at 1:2,000. Detection was carried out using ECL (Amersham Biosciences). β-Arrestin immunoblotting was carried out using A1CT rabbit polyclonal antibody at a dilution of 1:3,000 as previously described (4).

Immunoblotting for myocardial pERK was performed as previously described (5). Hearts were homogenized with Nonidet P-40 lysis buffer containing 137 mM NaCl, 20 mM Tris pH 7.4, 1% Nonidet P-40, 20% glycerol, 10 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF, 2.5 μg/ml aprotinin, and 2.5 μg/ml leupeptin.

Confocal Laser Microscopy. Confocal microscopy was performed as previously described (1). Briefly, HEK293 cells stably expressing FLAG-tagged WTβ_1AR and β_1AR mutants were transfected with cDNA encoding fluorescently labeled EGFR (i.e., EGFR-GFP, 2 μg). Following transfection, cells were trypsinized and plated onto 35-mm collagen-coated (5 μg/ml) glass-bottomed culture dishes (MatTek). Following stimulation, cells were washed once with PBS solution and fixed in 4% paraformaldehyde for 30 min. EGFR receptor internalization following stimulation was visualized by green fluorescence using a single sequential line of excitation filters. EGFR-GFP internalization was visualized using a combination of excitation of 488 nm and emission filters between 499 and 520 nm.

ICUE2 cAMP Assay. HEK293 cells stably overexpressing both the mouse β1AR and the cAMP biosensor ICUE2 were generated and validated as described (6). Intracellular cAMP concentrations were measured as a FRET ratio: the CFP intensity (438/24 excitation and 483/32 emission bandpass filters; Semrock) over FRET intensity (542/27 emission filter). Experiments were performed on a NOVOstar plate reader (BMG) as described previously (7). Data shown are the change in FRET ratio before and after addition of ligand.

Statistical Analysis. Data are expressed as mean $\pm$ SE. Statistical significance was determined by using one-way ANOVA with Bonferroni correction for multiple comparisons or Student unpaired t tests. A P value <0.05 was considered statistically significant.

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7. Wisler JW *et al.* (2007) A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. *Proc Natl Acad Sci USA* 104:16657–16662.

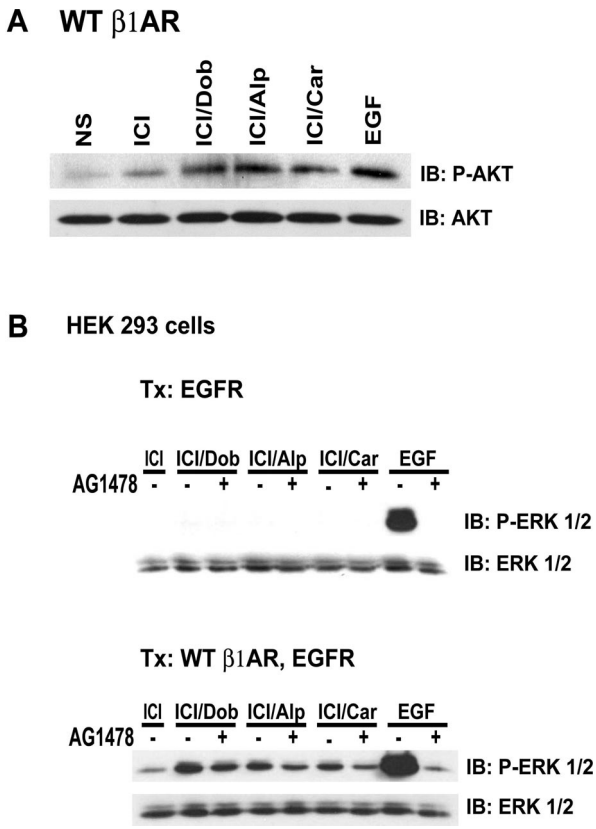

Fig. S1. Alp- or Car-stimulated ERK1/2 activation requires β_1AR expression and is sensitive to EGFR inhibition. (*A*) HEK 293 cells stably expressing WTβ_1ARs with transient transfection of FLAG-EGFR are treated with Dob, Alp, Car, or EGF. Both Alp and Car induced Akt activation. (*B*) HEK293 cells lacking endogenous β_1AR expression do not elicit phosphorylation of ERK1/2, whereas those transfected with WTβ_1ARs elicit an Alp- or Car-stimulated ERK response that is sensitive to EGFR inhibition. Tx, transient transfection.

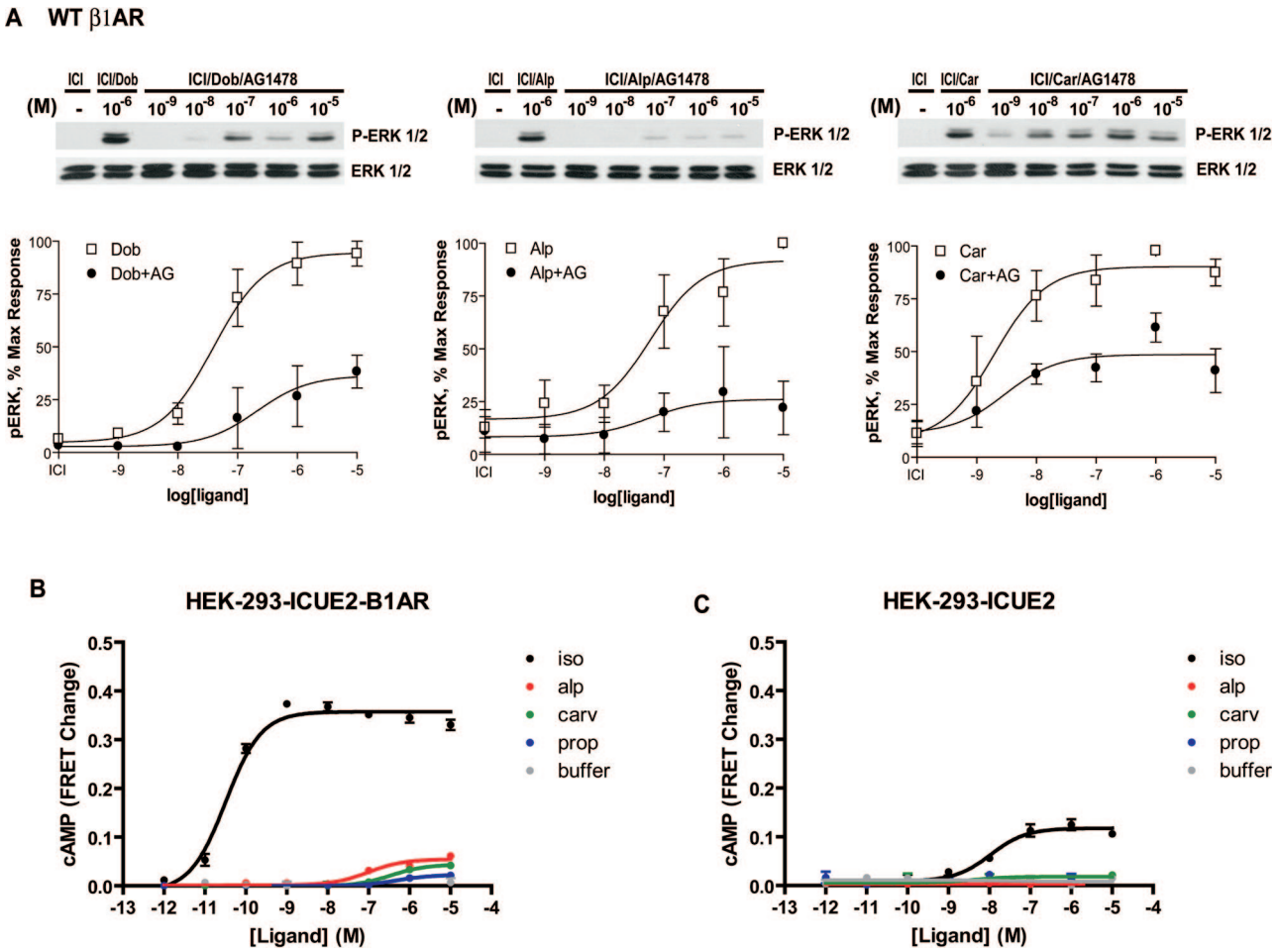

Fig. S2. Dose-dependent ERK responses and cAMP responses by Alp or Car. (*A*) Dose-dependent ERK responses by Alp or Car in the presence or absence of AG1478 (AG). HEK293 cells stably expressing WTβ_1ARs with transient transfection of FLAG-EGFR are treated with the indicated concentrations of Dob, Alp, or Car for 5 min. Whole-cell lysates were prepared and analyzed for pERK and ERK content by Western blot. Based on data in Fig. 2*C* in the main text, dose-response curves for ERK activation by Dob, Alp, or Car were redrawn as a percentage of maximal activity. Dose–response curves by Dob+AG, Alp+AG, or Car+AG were drawn as a relative percentage of observed activity in 1 μM of Dob, Alp, or Car. (*B* and *C*) Efficacy profiles of Alp and Car on G protein-dependent signaling pathway. cAMP responses are monitored by ICUE2 in HEK293 cells stably expressing β_1AR and the cAMP biosensor ICUE2 (*B*) and HEK 293 cells stably expressing the cAMP biosensor ICUE2 (*C*). Cells are treated for 3 min with a panel of ligands. cAMP agonism is measured as the rate of change of the ICUE2 FRET ratio corresponding to the rate of cAMP accumulation. Experiments are performed on a NOVOstar plate reader. Both Alp and Car show very slight activation of β_1AR G protein-dependent signaling in only the range from 1 to 10 μM. Data represent mean ± SE of at least five independent experiments.

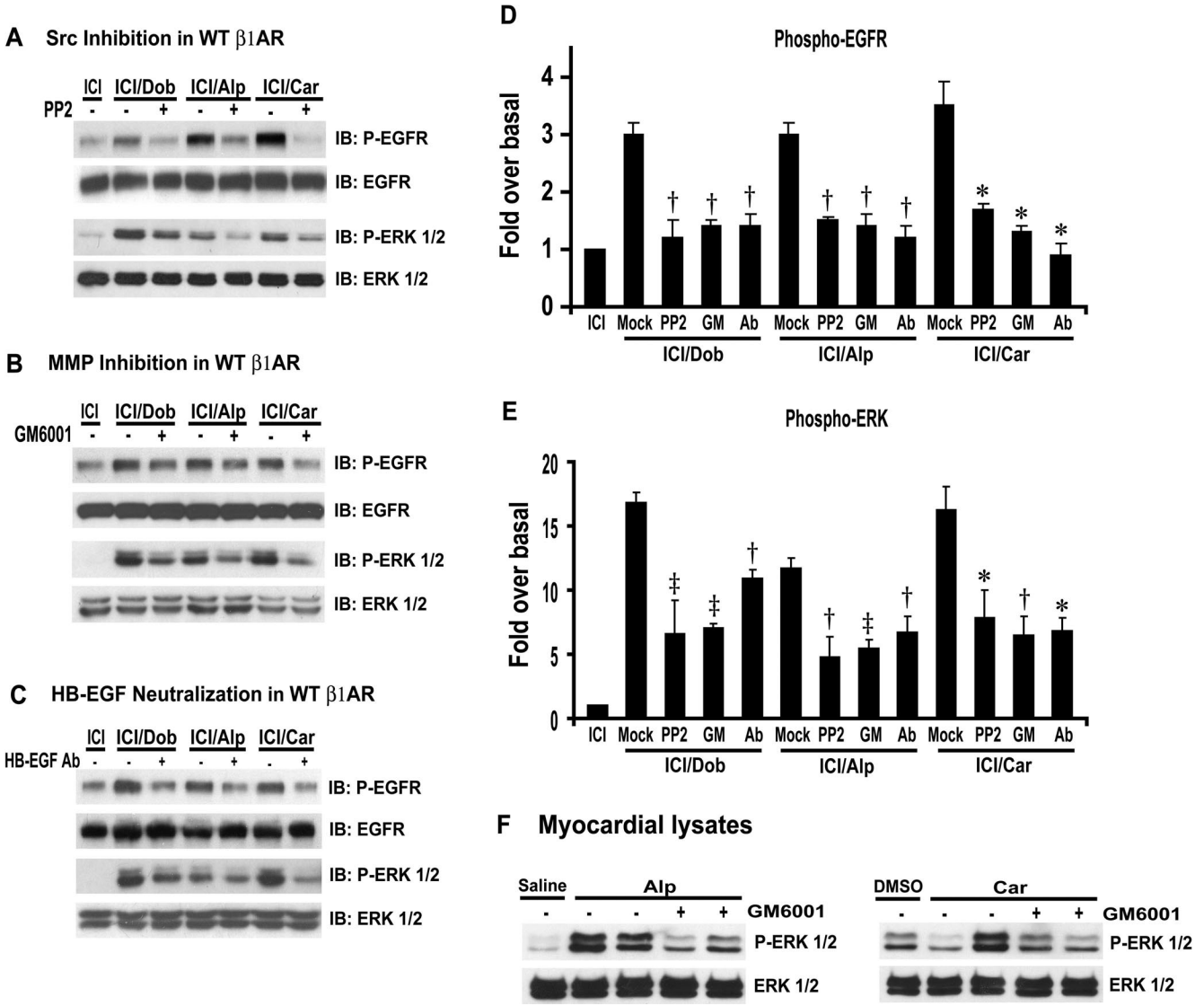

Fig. S3. Src, MMP, and HB-EGF are downstream of β-arrestin in β_1AR-mediated EGFR transactivation induced by Alp or Car. (*A–C*) HEK293 cells stably expressing WTβ_1AR are transfected with FLAG-EGFR. Pretreatment with inhibitors of Src (PP2) and MMP (ilomastat) and HB-EGF neutralizing antibody blocks Alp- or Car-induced EGFR transactivation. (*D* and *E*) Quantification of phospho-EGFR and phospho-ERK response. (*F*) WT mice were pretreated with ilomastat or DMSO followed by infusion with ligands. Hearts were removed after 5 min, and myocardial lysates were immunoblotted with anti-phospho-ERK1/2 and anti-total ERK1/2 antibodies. Data represent mean ± SE of at least three independent experiments. *, $P < 0.05$ vs. Mock, †, $P < 0.01$ vs. Mock, ‡, $P < 0.001$ vs. Mock.