MARCH2 promotes endocytosis and lysosomal sorting of carvedilol-bound β_2-adrenergic receptors

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Lysosomal degradation of ubiquitinated β_2-adrenergic receptors (β_2ARs) serves as a major mechanism of long-term desensitization in response to prolonged agonist stimulation. Surprisingly, the βAR antagonist carvedilol also induced ubiquitination and lysosomal trafficking of both endogenously expressed β_2ARs in vascular smooth muscle cells (VSMCs) and overexpressed Flag-β_2ARs in HEK-293 cells. Carvedilol prevented β_2AR recycling, blocked recruitment of Nedd4 E3 ligase, and promoted the dissociation of the deubiquitinases USP20 and USP33. Using proteomics approaches (liquid chromatography–tandem mass spectrometry), we identified that the E3 ligase MARCH2 interacted with carvedilol-bound β_2AR. The association of MARCH2 with internalized β_2ARs was stabilized by carvedilol and did not involve β-arrestin. Small interfering RNA–mediated down-regulation of MARCH2 ablated carvedilol-induced ubiquitination, endocytosis, and degradation of endogenous β_2ARs in VSMCs. These findings strongly suggest that specific ligands recruit distinct E3 ligase machineries to activated cell surface receptors and direct their intracellular itinerary. In response to β blocker therapy with carvedilol, MARCH2 E3 ligase activity regulates cell surface β_2AR expression and, consequently, its signaling.

Introduction

Agonist stimulation of cell surface seven-transmembrane G protein–coupled receptors (GPCRs or 7TMRs) leads to heterotrimeric G protein activation and second messenger–mediated cellular responses (Neves et al., 2002; DeWire et al., 2007). Immediately after their activation, 7TMRs are phosphorylated by GPCR kinases (GRKs) leading to the recruitment of cytosolic adaptors called β-arrestins, which terminate G protein signaling and initiate receptor endocytosis (Moore et al., 2007; Shenoy and Lefkowitz, 2011). 7TMR internalization is subsequently coupled to a second wave of signaling via the GRK–β-arrestin system (Reiter and Lefkowitz, 2006). Signal transduction at this stage is mostly regulated by postendocytic sorting mechanisms that cause either receptor degradation (signal termination) or receptor recycling (signal resensitization).

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Abbreviations used in this paper: 7TMR, seven-transmembrane G protein–coupled receptor; ANOVA, analysis of variance; β_2AR, β_2-adrenergic receptor; CHF, chronic heart failure; DTME, Dithio-bis-maleimidoethane; GPCR, G protein–coupled receptor; GRK, GPCR kinase; IP, immunoprecipitation; Iso, Isoproterenol; KO, knockout; LC-MS/MS, Liquid chromatography–tandem mass spectrometry; MDC, monodansylcadaverine; MEF, mouse embryo fibroblast; NS, nonstimulated control; P/S, penicillin/streptomycin; VSMC, vascular smooth muscle cell; WT, wild type.

7TMR trafficking is substantially influenced by dynamic ubiquitination and deubiquitination of the agonist-activated receptor (Shenoy, 2007; Shenoy and Lefkowitz, 2011). For the β_2-adrenergic receptor (β_2AR), agonist-induced ubiquitination by the HECT domain E3 ligase Nedd4 (neural precursor cell expressed developmentally down-regulated protein 4) is required for receptor trafficking to the lysosomes and subsequent receptor degradation (Shenoy et al., 2008). This process is counteracted by β_2AR deubiquitination, mediated by the deubiquitinases USP20 and USP33; deubiquitination commits the β_2AR to recycle and resensitize at the cell surface (Berthouze et al., 2009). These agonist-dependent processes tightly regulate the magnitude and duration of GPCR signal transduction, thus balancing the downstream cellular responses.

Activation of β_2ARs and α_1ARs in vascular smooth muscle cells (VSMCs) regulates vascular tone and directs blood flow to essential organs. Activation of cardiomyocyte βARs by catecholamines mediates the increase in heart rate and contractility

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 199 No. 5 817–830
www.jcb.org/cgi/doi/10.1083/jcb.201208192

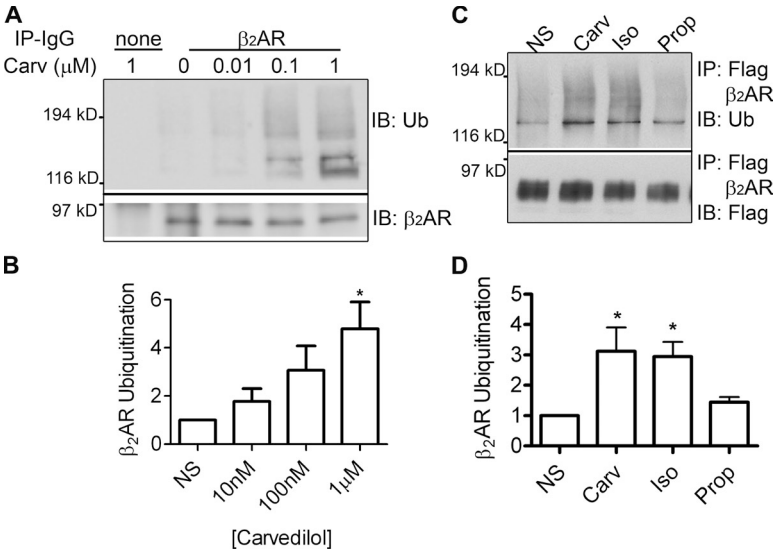

Figure 1. **Carvedilol induces β_2AR ubiquitination.** (A) Rat VSMCs were exposed to medium lacking or containing the indicated concentrations of carvedilol for 1 h and cell extracts were immunoprecipitated with either 2 µg normal rabbit IgG or anti-β_2AR polyclonal antibody (M-20) and probed with anti-ubiquitin (Ub; top) and anti-β_2AR (bottom) antibodies. (B) The ubiquitin signals in the immunoprecipitates were quantified and normalized to the respective β_2AR bands from four independent experiments and are plotted as the mean ± SEM. *, $P < 0.05$ compared with nonstimulated control (NS). (C and D) HEK-293 cells stably transfected with Flag-β_2AR were treated with carvedilol, Iso, propranolol (Prop), or vehicle (NS) for 1 h at 37°C; β_2AR immunoprecipitates were immunoblotted for ubiquitin (top) and β_2AR (bottom) and quantified as in B. *, $P < 0.05$ compared with NS ($n = 3$).

associated with stress or exercise. In chronic heart failure (CHF), catecholamine stimulation of βARs leads to pathological responses including myocyte apoptosis and hypertrophy (Xiao et al., 2004). In contrast, βAR antagonists (β blockers) that counteract the binding of catecholamines and block G protein signaling provide survival benefits to patients with CHF (Bristow, 2000). Recent studies have shown that the β blocker carvedilol has unique agonist properties in inducing βAR signaling specifically via β-arrestin while blocking G protein signaling, thus functioning as a β-arrestin–biased agonist (Wisler et al., 2007; Kim et al., 2008a; Shenoy, 2011).

Although carvedilol, metoprolol succinate, and bisoprolol fumarate are used for treating CHF (Hunt et al., 2009; Jabbour et al., 2010), some evidence suggests that the nonselective β blocker carvedilol possesses survival advantages over others (Louis et al., 2001; Domanski et al., 2003). In heart failure, both bisoprolol and metoprolol treatments cause an up-regulation of βAR expression, whereas carvedilol does not, despite being as effective as other β blockers in improving left ventricular function (Heilbrunn et al., 1989; Gilbert et al., 1996; Yamada et al., 1996; Flesch et al., 2001; Kindermann et al., 2004). Therefore, carvedilol could be mechanistically unique in initiating specific itineraries for receptor trafficking and regulating βAR expression as well as signaling. Herein, we report a hitherto unknown molecular mechanism of carvedilol-induced β_2AR endocytosis and down-regulation promoted by a novel interaction with an E3 ubiquitin ligase, MARCH2 (membrane-associated RING-CH2).

Results

The β blocker carvedilol induces β_2AR ubiquitination and promotes lysosomal trafficking

Because the β_2AR agonist isoproterenol (Iso) induces ubiquitination of the receptor (Shenoy et al., 2001, 2008; Liang and Fishman, 2004; Berthouze et al., 2009; Xiao and Shenoy, 2011), one would expect β blockers to function as antagonists and block this effect. Contrary to this premise, the β blocker carvedilol induced dose-dependent ubiquitination of the β_2AR in VSMCs, as can be seen with β_2AR immunoprecipitation (IP; Fig. 1, A and B). To corroborate the identity of the β_2AR as the immunoprecipitated, carvedilol-responsive protein species from VSMCs, we took several approaches. First, the reactivity of the anti-β_2AR IgG toward either purified recombinant β_2AR or to endogenously expressed receptor protein was eliminated by preblocking the antibody with purified β_2AR protein (Fig. S1 A). Second, transfection of β_2AR-specific siRNA decreased the immunoblotted signals for endogenous β_2AR by 55% in rat VSMCs (Fig. S1 B). Third, we obtained equivalent results with epitope-tagged β_2ARs overexpressed in HEK-293 cells, challenged with either the antagonist carvedilol or the agonist Iso, but not the antagonist propranolol (Fig. 1, C and D). Thus, although their SDS-PAGE migration differs from the polydisperse, hyperglycosylated β_2AR bands detected in overexpression systems (Fig. 1, A and C, compare β_2AR blots), the sharp bands detected in the immunoprecipitated samples from VSMCs are authentic β_2AR bands. Detection of similarly "sharp" bands for endogenously expressed β_1ARs has been previously shown in cardiac extracts isolated from wild-type (WT) but not β_1AR knockout (KO) mice (Rohrer et al., 1996). Thus, the β blocker carvedilol, used to treat CHF, acts at the β_2AR to induce ubiquitination in a manner similar to the agonist Iso but completely distinct from the antagonist propranolol.

To determine the fate of β_2ARs ubiquitinated in a carvedilol-responsive manner, we labeled cell surface endogenous β_2ARs in quiescent cells using an IgG (I3D6) that recognizes β_2AR extracellular domains. We then traced the intracellular destination of the I3D6-bound β_2ARs after treatment with different ligands. This approach proved practicable with endogenous β_2ARs in VSMCs (Fig. 2, A and B; and Fig. S1, C and D), as well as with overexpressed Flag-β_2ARs in HEK-293 cells (Fig. 2 C). Upon stimulation with either carvedilol or Iso, β_2ARs internalize and colocalize with the LysoTracker dye that labels late endosomes and lysosomes (Fig. 2, A–C; and Fig. S1 D). These colocalization patterns were confirmed by determining Pearson's correlation

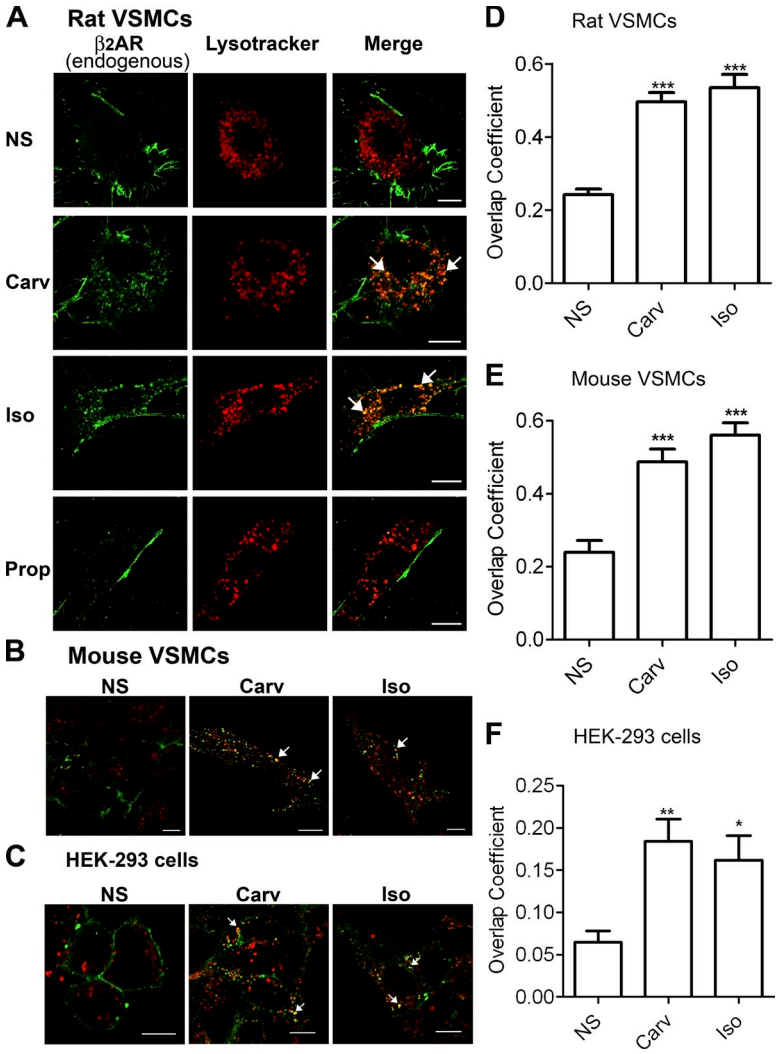

Figure 2. **Carvedilol induces lysosomal trafficking of the β_2AR.** Early passage rat (A) or mouse (B) VSMCs were incubated at room temperature for antibody surface labeling (I3D6-β_2AR) along with LysoTracker red. After 1 h uptake, the antibody was washed out and cells were stimulated at 37°C for 1 h with 1 µM carvedilol, Iso, or propranolol (Prop) diluted in serum-free media. At the end of stimulation, cells were fixed, permeabilized, and labeled with a secondary antibody conjugated to Alexa Fluor 488. Images were obtained with a confocal microscope and a 100× oil objective. (C) Lysosomal trafficking of overexpressed Flag-β_2ARs in HEK-293 cells assessed by the same procedure used in A and B. Bars, 10 µm. Arrows indicate colocalization of receptors and LysoTracker dye. (D–F) The bar graphs (mean ± SEM) represent Pearson's correlation coefficients that were calculated for β_2AR and LysoTracker Red colocalization in the respective cells for NS, carvedilol and Iso-stimulated conditions and were significant, as determined by one-way ANOVA (***, $P < 0.001$; ** $P < 0.01$; *, $P < 0.05$ versus NS; $n > 20$ cells for all conditions).

coefficients, which were significantly increased above basal signals upon either carvedilol or Iso stimulation (Fig. 2, D–F). Propranolol, which does not evoke β_2AR ubiquitination, did not induce any β_2AR internalization (Fig. 2 A). As was the case with carvedilol-induced β_2AR ubiquitination, carvedilol-induced β_2AR lysosomal trafficking was dose dependent (Fig. S2 A). Additionally, carvedilol-induced effects were completely blocked upon pretreatment with the β_2AR-specific antagonist ICI 118,551 (Fig. S2 B). Overall, carvedilol induced trafficking of β_2ARs with similar efficiency and comparable kinetics in both HEK-293 (Fig. 2, C and F; and Fig. S2 C) and VSMCs (Fig. 2, A, B, D, and E).

Effect of endocytosis inhibitors and lysine mutations on carvedilol-induced internalization and ubiquitination

Generally, agonist-induced internalization of the β_2AR involves clathrin- and dynamin-dependent mechanisms (Zhang et al., 1996; Gagnon et al., 1998; Ahn et al., 1999; Pierce et al., 2000; Shenoy and Lefkowitz, 2003). To examine if carvedilol-induced internalization proceeds via these trafficking mechanisms, we treated VSMCs and HEK-293 cells with monodansylcadaverine (MDC), which inhibits receptor clustering into clathrin-coated pits (Haigler et al., 1980; Nandi et al., 1981; Phonphok and Rosenthal, 1991), and dynasore, which inhibits dynamin GTPase activity and dynamin-dependent endocytosis (Macia et al., 2006). We then determined the amount of cell surface receptor by surface antibody labeling followed by ELISA (see Materials and methods). Both MDC and dynasore inhibited β_2AR endocytosis induced by Iso (Fig. 3, A–C). In contrast, β_2AR endocytosis induced by carvedilol was inhibited by dynasore, but not MDC (Fig. 3, A–C). Thus, carvedilol-induced β_2AR endocytosis appears to involve clathrin-independent yet dynamin-dependent internalization mechanisms (Fig. 3, A–C).

Both MDC and dynasore augmented the levels of ubiquitinated β_2ARs seen after 1 h of Iso stimulation; however, only dynasore exerted this effect on β_2ARs in carvedilol-challenged cells (Fig. 3 D). These data suggest that both Iso and carvedilol promote ubiquitination of the β_2AR at the plasma membrane: if ubiquitination occurred after internalization, endocytosis inhibitors would engender a decline in ubiquitinated species, rather than the increase we observed.

Previous studies indicate that Iso-stimulated ubiquitination and lysosomal degradation are ablated in a β_2AR mutant in which all Lys residues are mutated to Arg (0K-β_2AR; Shenoy et al., 2001; Liang et al., 2008; Xiao and Shenoy, 2011), even though

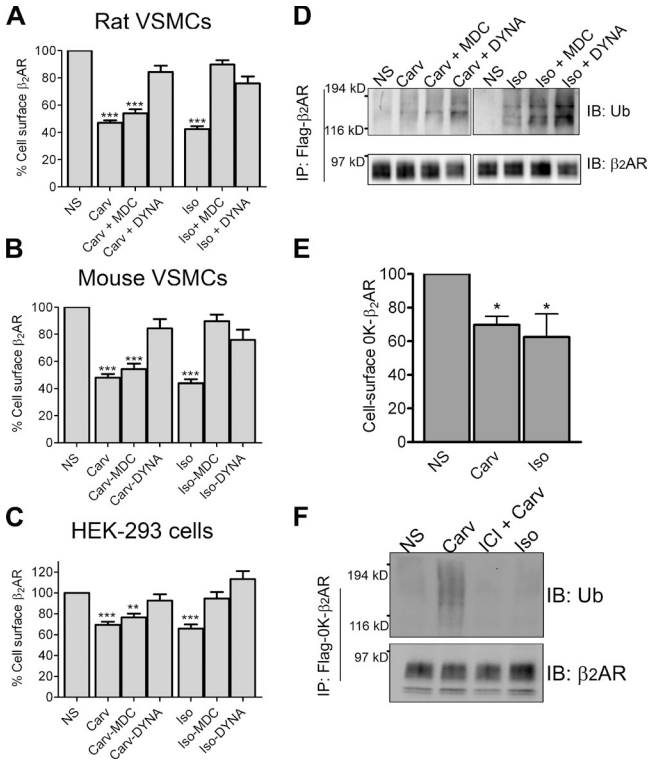

Figure 3. **Effects of endocytosis inhibitors and lysine mutations on carvedilol-induced internalization and ubiquitination.** (A) Rat VSMCs were prelabeled with I3D6-β_2AR antibody at 4°C and subsequently stimulated with vehicle, carvedilol, or Iso for 1 h at 37°C ± indicated inhibitors (100 µM monodansyl cadaverine [MDC] or 80 µM Dynasore [DYNA]). The amount of antibody remaining at the cell surface was quantified by ELISA. Bar graphs show percentage of cell surface receptors (mean ± SEM) from four independent experiments where receptors in unstimulated cells equal 100%. Similar data obtained for the internalization of endogenous β_2ARs in mouse VSMCs and overexpressed Flag-β_2ARs in HEK-293 cells are shown in B and C, respectively. Compared with NS: ***, $P < 0.001$; **, $P < 0.01$. (D) Carvedilol- and Iso-induced ubiquitination of Flag-β_2AR in HEK-293 cells was determined as in Fig. 1. Indicated inhibitors were added 5 min before stimulation. Blots shown are representative of similar results in three independent experiments. (E) Internalization of Flag–0K-β_2AR as induced by carvedilol or Iso was determined by cell surface antibody labeling and ELISA and shown as mean ± SEM. Compared with NS: *, $P < 0.05$. (F) Flag–0K-β_2AR was immunoprecipitated after indicated treatments and probed for ubiquitination (top). The same blot was reprobed for receptor levels (bottom). Data shown are representative of similar results from three independent experiments.

Iso-induced internalization of the 0K-β_2AR into endosomes is not affected (Shenoy et al., 2001; Berthouze et al., 2009). As shown in Fig. 3 E, both carvedilol and Iso induced significant internalization of Flag–0K-β_2ARs from the cell surface. In accord with previous studies, Iso failed to induce ubiquitination of the 0K-β_2AR (Fig. 3 F). Surprisingly, however, carvedilol did induce ubiquitination of the 0K-β_2AR (Fig. 3 F). This carvedilol-induced ubiquitination was blocked by pretreatment of the cells with ICI 118,551 (Fig. 3 F). These data suggest that carvedilol induces a β_2AR conformation that allows ubiquitination of noncanonical β_2AR sites. Ubiquitination generally targets lysyl residues, but cysteinyl and rarely seryl or threonyl residues can also be appended with ubiquitin moieties (Cadwell and Coscoy, 2005; Herr et al., 2009), which could explain carvedilol-induced ubiquitination of the 0K-β_2AR.

Carvedilol stimulation blocks recruitment of Nedd4

Agonist-induced lysosomal degradation of the β_2AR follows its ubiquitination by the E3 ubiquitin ligase Nedd4 (Shenoy et al., 2008). Consistent with this earlier finding, Iso stimulation increased the association of endogenous Nedd4 with the β_2AR (Fig. 4, A and B). In contrast, carvedilol treatment dramatically diminished the association of Nedd4 with the β_2AR (Fig. 4, A and B). To test whether Nedd4 effected carvedilol-induced β_2AR degradation, we used Nedd4 RNAi in VSMCs. In the presence of cycloheximide that inhibits protein synthesis, carvedilol promoted significant degradation of the β_2AR within 6–24 h, and this degradation was not affected by Nedd4 knockdown (Fig. 4, C–E). In contrast, Iso-stimulated degradation was completely blocked upon Nedd4 knockdown in VSMCs (Fig. 4, F–H). These data strongly suggest that carvedilol can induce degradation of β_2AR in VSMCs through mechanisms independent of Nedd4.

Carvedilol stimulation promotes dissociation of deubiquitinases and blocks receptor recycling

Ubiquitination of the β_2AR is reversed by the deubiquitinases USP20 and USP33, which tonically associate with the β_2AR (Berthouze et al., 2009). Because carvedilol enhanced β_2AR ubiquitination and degradation independently of the known β_2AR E3 ligase Nedd4, we reasoned that carvedilol might diminish β_2AR deubiquitination by reducing the association of the β_2AR with USP20 and/or USP33. To test this hypothesis, we assayed β_2AR/USP association by coIP. Whereas USP20 and USP33 showed the expected stable association with the Iso-bound β_2AR, they both dissociated from the carvedilol-bound β_2AR (Fig. 5, A–D). Because USP20/33-mediated β_2AR deubiquitination regulates recycling of internalized β_2ARs (Berthouze et al., 2009), we asked whether this process was also affected by carvedilol. Cells were labeled with anti-β_2AR (I3D6) IgG, challenged with either Iso or carvedilol for 1 h, washed, and then allowed to recover for 1 h at 37°C. Subsequent confocal microscopy revealed that both Iso and carvedilol challenged engendered β_2AR internalization. However, whereas Iso-treated cells showed complete recovery of cell surface β_2ARs upon inducing recycling, carvedilol-treated cells showed persistent internalization of the majority of β_2ARs (Fig. 5 E). To complement these confocal microscopy studies, we also assayed cell surface β_2ARs by ELISA for the extracellular domain anti-β_2AR IgG. As shown in Fig. 5 F, both carvedilol and Iso induced β_2AR internalization, but only Iso stimulation caused recycling of the internalized receptors. Together, these data indicate major differences between Iso- and carvedilol-triggered β_2AR trafficking and protein–partner interaction, even though both ligands induce β_2AR ubiquitination and endocytosis.

Agonist- and β blocker–induced trafficking involve different E3 ubiquitin ligases

Because Nedd4 is not involved in β_2AR trafficking (Fig. 4), we hypothesized that the carvedilol-bound conformation of the β_2AR might engage novel protein partners to mediate ubiquitination

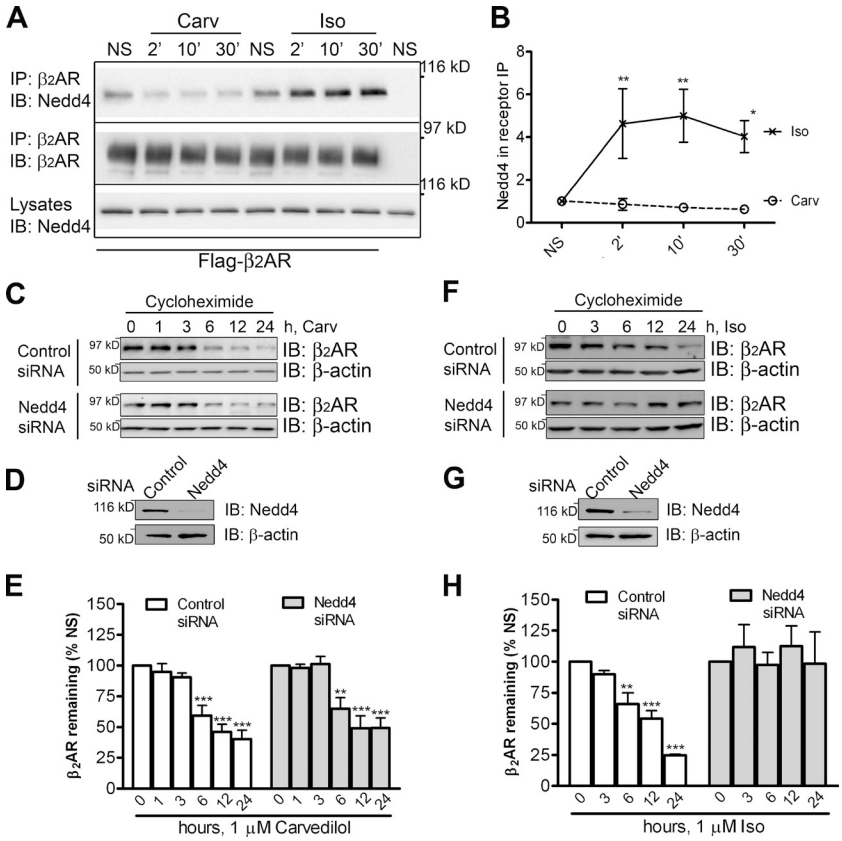

Figure 4. **Carvedilol-induced β_2AR degradation is not mediated by Nedd4.** (A) Flag-β_2ARs were immunoprecipitated from HEK-293 cells after indicated treatments and chemical cross-linking with DTME. The amounts of Nedd4 (top) and β_2AR (middle) in each IP sample is shown as detected by the respective antibodies. The bottom panel shows equivalent amounts of endogenous Nedd4. The rightmost lane is a control sample prepared from cells that do not express Flag-β_2AR. (B) The amounts of Nedd4 in the IP samples were quantified and normalized to the β_2AR levels and plotted as mean ± SEM from four independent experiments. *, $P < 0.05$; **, $P < 0.01$. Rat VSMCs were transfected with siRNA targeting no mRNA (Control) or Nedd4 and treated with 1 μM carvedilol (C) or Iso (F) in the presence of protein synthesis inhibitor cycloheximide (20 μM) for the indicated times. Equal amounts of whole cell extracts were immunoblotted for endogenous β_2AR and subsequently the blots were stripped and reprobed for β-actin (bottom). The extent of Nedd4 knockdown is shown in D and G. (E and H) β_2AR protein bands were quantified and normalized to respective actin levels and plotted as bar graphs. The amount of β_2AR in NS cells represents 100% in each set of samples. Data shown are mean ± SEM from five independent experiments. Indicated samples were significantly decreased as compared with respective NS samples; one-way ANOVA, ***, $P < 0.001$; **, $P < 0.01$. Additionally, the control and Nedd4 siRNA groups were significantly different only for Iso-stimulated samples as analyzed by two-way ANOVA.

and subsequent lysosomal trafficking. To identify β_2AR interacting proteins that specifically recognize Iso- or carvedilol-induced conformations of the β_2AR, we isolated Flag–β_2AR complexes from HEK-293 cells that were treated with vehicle, Iso, or carvedilol for 6 h; fractionated samples on SDS-PAGE; trypsin digested the proteins in gel slices; performed liquid chromatography–tandem mass spectrometry (LC-MS/MS) on the digested peptides; and analyzed the data sets by Mascot (Xiao et al., 2007). By this approach, we identified a candidate E3 ubiquitin ligase, MARCH2, as well as several other protein partners that interact with the β_2AR only upon carvedilol treatment (Fig. S3 A).

Carvedilol recruits MARCH2 to promote ubiquitination and internalization of cell surface β_2ARs

In unstimulated cells, MARCH2-GFP is mostly distributed in the cytoplasmic compartment and some expression is detected at the plasma membrane (Fig. 6 A). However, we detected a small amount of colocalization of β_2AR and MARCH2 at the plasma membrane in unstimulated cells. Carvedilol challenge markedly increased MARCH2/β_2AR colocalization as both proteins translocated to endosomes (Fig. 6 A and Fig. S3 B). In IP assays, MARCH2 and the β_2AR showed constitutive association that was augmented by carvedilol treatment (Fig. 6 B). A similar carvedilol-induced increase in association was also detected between HA-MARCH2 and β_2AR (Fig. S3, B and C). In contrast, Iso did not promote colocalization of MARCH2 and β_2AR (Fig. 6 A and Fig. S3 B). Unfortunately, we could not detect MARCH2 protein at endogenous levels of expression despite using four different commercially available MARCH2 antibodies (see Materials and methods) and three custom-generated ones (Nakamura et al., 2005)—all of which detected overexpressed MARCH2.

To evaluate whether MARCH2 E3 ligase activity affects carvedilol-induced binding with the β_2AR and to determine if MARCH2 catalytic activity is required for its cotrafficking with the receptor, we generated and tested a MARCH2 mutant ($MARCH2^{CCH}$). This construct carries mutations within the RING-CH domain (at cysteines 64 and 67 as well as histidine 90) that are critical for coordinating zinc ions (Fig. 7 A). Alteration of the zinc coordinating residues or deletion of the RING domain generally ablates enzymatic activity of RING domain E3 ligases (Joazeiro and Weissman, 2000). To assess if WT and the putative catalytically inactive MARCH2 would have differences in their subcellular distributions, we examined WT MARCH2 and $MARCH2^{CCH}$ for their colocalization with the early endosomal marker (early endosomal antigen 1) and LysoTracker. As shown in Fig. S4, both WT and mutant constructs colocalized similarly with these vesicular markers and this did not change with carvedilol stimulation.

The mutant $MARCH2^{CCH}$ showed a significant increase in carvedilol-stimulated interaction with the β_2AR within 2 min after stimulation (Fig. 7 B) and the complexes remained stable for 30 min (Fig. 7, B and C), as they did for the WT MARCH2 (Fig. 6 B). In contrast, Iso triggered only a weak association between the β_2AR and $MARCH2^{CCH}$ (Fig. 7, B and C). $MARCH2^{CCH}$ overexpression prevented β_2AR ubiquitination after carvedilol

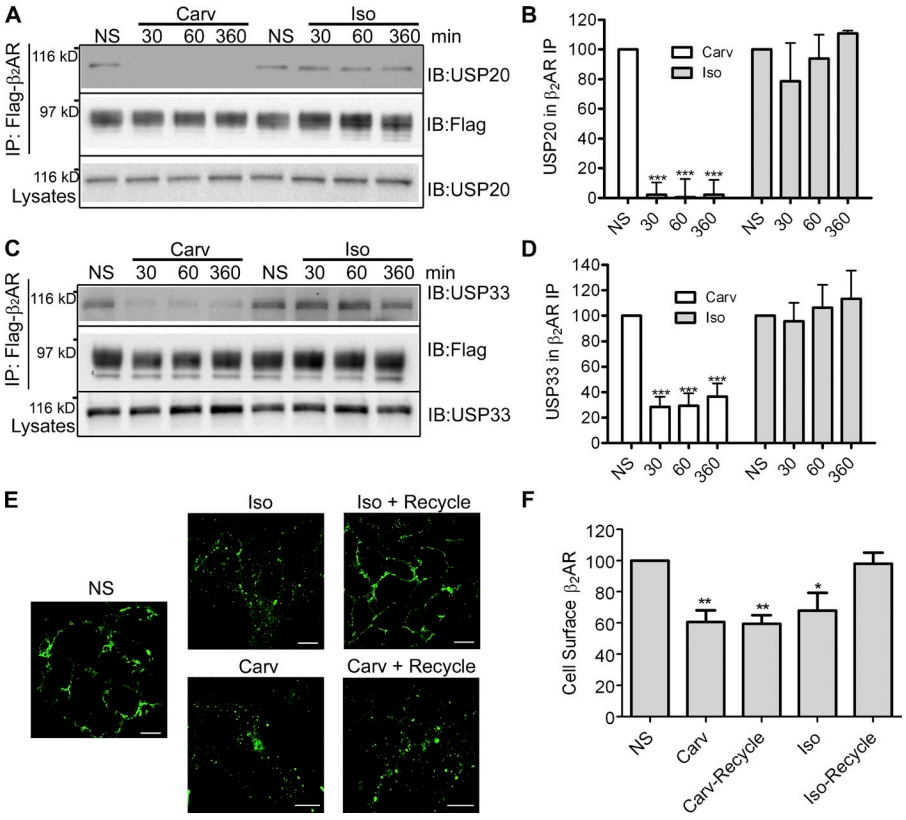

Figure 5. **Carvedilol prevents DUB-β_2AR binding as well as β_2AR recycling.** (A) After overnight serum starvation, cells with stable Flag-β_2AR expression were stimulated by either 1 µM carvedilol or Iso for the indicated times. Flag-β_2AR immunoprecipitates were then immunoblotted serially for USP20 and the β_2AR. The lysate expression levels (5% of cell extracts used in the IP) are shown in the bottom panel. (B) The bar graphs were obtained by calculating the ratio between the endogenous ubiquitin-specific protease and β_2AR signals for each time point. The result is the mean ± SEM of three independent experiments; ***, $P < 0.001$ versus NS sample. (C and D) The experiments from A and B were repeated, but β_2AR immunoprecipitates were probed for USP33 instead of USP20. (E) Confocal images of cells stained with 13D6 IgG depict the Flag-β_2AR in untreated cells at the plasma membrane (left; NS); in endocytic vesicles after 1 h at 37°C with either Iso (middle top) or Carv (middle bottom); and after removal of I3D6 IgG, washing with culture medium, and further incubation (1 h, 37°C) to promote recycling (right, top and bottom). Recycling is observed only in cells that were previously treated with Iso. Bars, 20 µm. (F) Cell surface ELISA for the I3D6 IgG (labeling at 4°C; see Materials and methods) was used to quantitate cell surface Flag-β_2ARs, which were normalized to those present on NS cells. Plotted are the means ± SEM from three independent experiments: **, $P < 0.01$; *, $P < 0.05$ versus NS.

stimulation, whereas WT MARCH2 overexpression promoted β_2AR ubiquitination to the same extent as endogenous MARCH2 (Fig. 7, D and E). Contrastingly, Iso-stimulated ubiquitination was unaffected by WT MARCH2, but augmented by MARCH2^{CCH} (Fig. 7, D and E). These data suggest that a low level of association of MARCH2 and agonist-activated β_2ARs occurs, and removal of this "inhibitory" MARCH2 activity (by overexpression of MARCH2^{CCH}) likely potentiates Nedd4-mediated ubiquitination of agonist-bound receptors.

Consistent with the binding and ubiquitination studies shown in Fig. 7 (B–E), stimulation with carvedilol, but not Iso, engendered MARCH2^{CCH}/β_2AR colocalization at the plasma membrane, and the protein complexes remained there even at 60 min after carvedilol treatment (Fig. 7 F). Thus, coexpression of MARCH2^{CCH} inhibited both ubiquitination and internalization of carvedilol-bound β_2ARs. In marked contrast to the effect of MARCH2^{CCH} on β_2AR endocytosis by carvedilol (Fig. 7 F), WT MARCH2 coexpression promoted robust internalization of the β_2AR at 60 min of carvedilol stimulation (Fig. 7 G). This suggests that carvedilol-induced ubiquitination mediated by MARCH2 is critical for promoting β_2AR endocytosis. These data also suggest that MARCH2 catalytic activity is not required for its interaction with the β_2AR and loss of activity creates a "substrate trap" leading to a stable complex formation between the two molecules.

MARCH2 functions as a critical regulator of carvedilol-induced ubiquitination and lysosomal degradation

To assess the physiological relevance of MARCH2-mediated effects on the β_2AR, we used siRNA-mediated knockdown of endogenously expressed MARCH2. Three different siRNA oligonucleotides targeted to mRNA sequences conserved in both mouse and rat were tested in VSMCs isolated from both species and each led to a significant decrease in mRNA levels (MARCH2-1: >80%; MARCH2-2 and MARCH2-3: 50–70% decrease; Fig. 8 A and Fig. S5 A). Upon depletion of MARCH2, carvedilol-induced β_2AR ubiquitination (Fig. 8, B and C; and Fig. S5 B) was abolished in both rat and mouse VSMCs. In addition, carvedilol-induced internalization of the β_2AR was also completely eliminated (Fig. 8, D and E; and Fig. S5, C and D), reiterating the link between MARCH2-induced ubiquitination and β_2AR endocytosis. To determine if MARCH2 knockdown affects β_2AR degradation, we performed degradation assays in the presence of cycloheximide and assessed protein levels after 6 and 24 h of carvedilol stimulation as shown in Fig. 4 C. Additionally, to confirm that these effects are specific for MARCH2 activity, we performed siRNA rescue by transfecting a plasmid encoding human MARCH2 cDNA into rat or mouse VSMCs along with the siRNA. Carvedilol-induced β_2AR degradation was blocked upon down-regulating MARCH2 (Fig. 8, F and G; and Fig. S5, E and F). Furthermore, rescue of MARCH2 expression by cDNA transfection reversed this effect of MARCH2 knockdown and significant β_2AR degradation was detected (Fig. 8, F and G; and Fig. S5, E and F). These data provide convincing evidence that MARCH2 mediates carvedilol-induced ubiquitination of endogenous β_2ARs in VSMCs and that this ubiquitination is required for β_2AR endocytosis and trafficking to the lysosomes to promote degradation of the β_2AR protein.

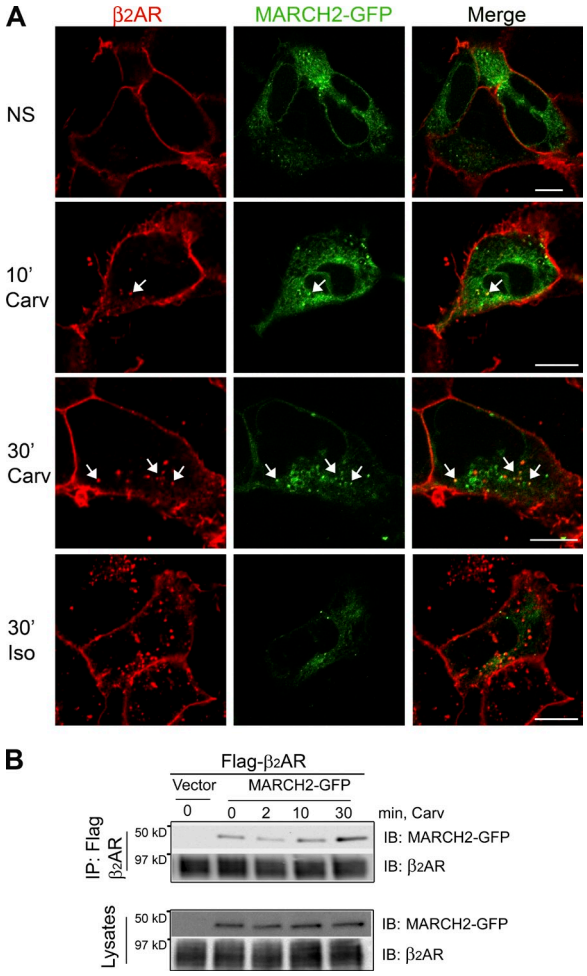

Figure 6. **β blocker carvedilol potentiates MARCH2/β_2AR binding on endocytic vesicles.** (A) HEK-293 cells expressing Flag-β_2ARs were transfected with MARCH2-GFP, stimulated with carvedilol or Iso for the indicated times, and immunostained for the β_2AR. Confocal panels show subcellular distribution of Flag-β_2AR (red) and MARCH2-GFP (green) alone or together (Merge). Colocalization of β_2AR and MARCH2 is observed at both 10 and 30 min after carvedilol stimulation in small vesicles (arrows). Bars, 10 µm. Results are representative of images obtained from four independent transfections. (B) HEK-293 cells with stable expression of Flag-β_2AR were transfected with either pEGFP vector or MARCH2-GFP and Flag immunoprecipitates were analyzed for bound MARCH2 after carvedilol stimulation for different times. The results shown are representative of three independent experiments.

Role of β-arrestins

Carvedilol was previously shown to induce transient β-arrestin2–GFP recruitment to a chimeric β_2AR-V_2 vasopressin receptor (Wisler et al., 2007). Carvedilol but not propranolol promoted Flag-β_2AR internalization in HEK-293 cells, but the role of β-arrestin in this process was not determined (Wisler et al., 2007). Furthermore, our previous studies have shown that β-arrestin2 is an essential adaptor for mediating agonist-dependent ubiquitination of the β_2AR as it binds and recruits Nedd4 to the β_2AR in an agonist-dependent manner (Shenoy et al., 2001, 2008). To determine whether β-arrestins are required for carvedilol-induced ubiquitination and trafficking of the β_2AR, we used β-arrestin1/2 double KO mouse embryo fibroblasts (MEFs; Kohout et al., 2001). In these β-arrestin1/2 KO MEFs β_2AR ubiquitination was promoted by carvedilol and blocked by pretreatment with ICI 118,551 (Fig. 9, A and B). Neither β-arrestin1 nor 2 rescue altered the carvedilol-induced β_2AR ubiquitination detected in these KO MEFs (Fig. 9, C and D). Moreover, although Iso-stimulated β_2AR internalization is not observed in these cells (Kohout et al., 2001), we could detect β_2AR internalization upon carvedilol stimulation (Fig. 9 E). The internalized β_2AR was colocalized with the LysoTracker dye, indicating that the internalized carvedilol-bound β_2AR is sorted to lysosomes (Fig. 9, E and F). In these β-arrestin1/2 double KO MEFs, both WT MARCH2 and MARCH2^{CCH} were recruited to the β_2AR upon carvedilol stimulation with kinetics similar to those observed in HEK-293 cells, which express normal amounts of endogenous β-arrestins (Figs. 6 B, 7 B, and 9 G). Together, these results suggest that unlike the agonist-induced effects on the β_2AR, which are dependent on β-arrestin expression, carvedilol-induced MARCH2/β_2AR association, β_2AR ubiquitination, internalization, and lysosomal trafficking occur in the absence of β-arrestins.

Together with previous studies, this work supports a paradigm in which carvedilol can evoke parallel and independent molecular effects by binding to the β_2AR (Fig. 10). These effects include blockade of G protein coupling (Ruffolo et al., 1990), stimulation of β-arrestin–dependent signaling (Wisler et al., 2007), and induction of MARCH2-mediated β_2AR ubiquitination and subsequent lysosomal degradation. Moreover, carvedilol-induced β_2AR internalization requires MARCH2-mediated ubiquitination of the β_2AR, suggesting that β blockers engage ubiquitin as a signal for β_2AR endocytosis.

Discussion

We report the molecular and cellular effects of a clinically relevant β blocker, carvedilol, on its pharmacologic target β_2AR in a physiologically relevant cell system. Our data reveal a novel molecular mechanism by which carvedilol (a βAR antagonist that reduces mortality in CHF) induces persistent βAR downregulation. Previous findings have shown that acute treatments with carvedilol stimulate GRK6-mediated receptor phosphorylation of the β_2AR- and β-arrestin2–dependent MAPK signaling in HEK-293 cells (Wisler et al., 2007; Kim et al., 2008a; Nobles et al., 2011). In the current work, we demonstrate that prolonged treatment with carvedilol causes lysosomal trafficking and degradation of endogenous β_2AR in primary VSMCs. Further, carvedilol-induced internalization occurs independent of β-arrestin binding and proceeds via clathrin-independent, yet dynamin-dependent, mechanisms. We have also discovered that carvedilol-bound β_2AR is a physiological substrate for the E3 ubiquitin ligase MARCH2, and the resulting ubiquitin modification of the receptor is required for both internalization and lysosomal sorting of the β_2AR.

Carvedilol-induced ubiquitination pattern of both endogenous and Flag-β_2ARs are similar and detected as high molecular mass bands as reported before for agonist-stimulated ubiquitination (Shenoy et al., 2001). Because both Iso- and carvedilol-induced ubiquitination are detectable with the anti-ubiquitin antibody clone FK1 that preferentially detects polyubiquitin chains (Fujimuro and Yokosawa, 2005), both Iso and carvedilol lead

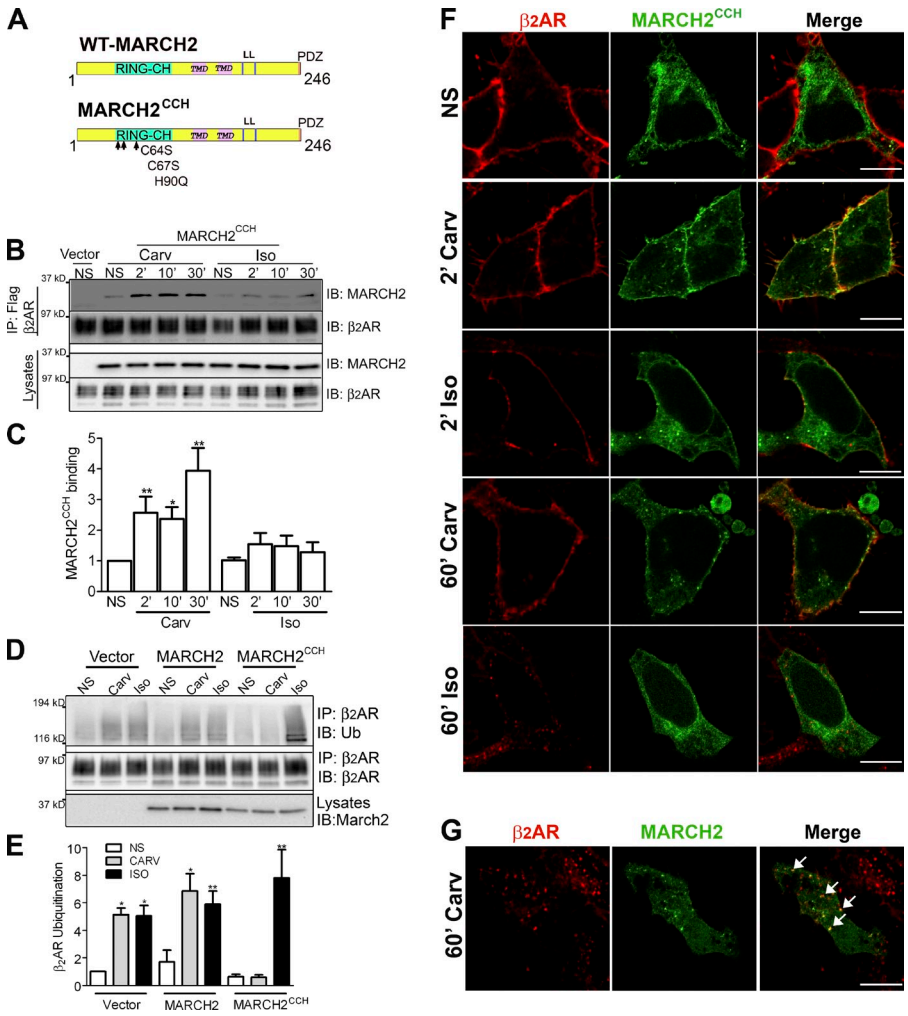

Figure 7. **A catalytically inactive MARCH2 mutant binds β_2AR and blocks carvedilol-induced ubiquitination and internalization.** (A) Schematic of WT and mutant MARCH2 constructs. RING-CH, transmembrane domains (*TMD*), dileucine (LL) motifs, and PDZ-interacting domains are indicated. In the MARCH2^{CCH} mutant, three residues within the catalytic domain are mutated: Cys64 and Cys67 to Ser and His90 to Gln. (B) HEK-293 cells with stable expression of Flag-β_2AR were transfected with either pCDNA3 vector or HA-MARCH2^{CCH} and Flag immunoprecipitates were analyzed for bound MARCH2 after carvedilol or Iso stimulation for different times. (C) The signals for MARCH2 in β_2AR immunoprecipitates were normalized to the respective β_2AR amounts in the IP and plotted as mean ± SEM of five independent experiments. **, $P < 0.01$; *, $P < 0.05$ versus NS sample. (D) HEK-293 cells with stable expression of Flag-β_2AR were transfected with either WT MARCH2 or MARCH2^{CCH} and challenged ±1 µM carvedilol or Iso stimulation for 1 h, and then solubilized and analyzed for ubiquitination. Flag-β_2AR immunoprecipitates were immunoblotted serially for ubiquitin (top) and the β_2AR (middle). Cell lysates were immunoblotted for MARCH2 (bottom). (E) The bar graph presents β_2AR ubiquitination normalized to cognate β_2AR band density as mean ± SEM quantified from three independent experiments. **, $P < 0.01$; *, $P < 0.05$ versus NS sample from vector transfection. (F) The confocal panels show the subcellular distribution of Flag-β_2AR (red) and MARCH2^{CCH}-GFP (green) alone or together (Merge) in quiescent cells and after stimulation with either 1 µM carvedilol or Iso for 2 or 60 min. Colocalization of β_2AR and MARCH2 is observed at the plasma membrane only for 2 min with carvedilol and internalized receptors are not detected after 60 min with carvedilol. (G) Confocal images show robust internalization of Flag-β_2AR and colocalization (arrows) with WT MARCH2-GFP after 60-min carvedilol treatment. Bars, 10 µm.

to polyubiquitination of the β_2AR. We recently reported that Iso-induced β_2AR ubiquitination involves 5 of the 14 intracellular lysines located in the third intracellular loop and carboxyl tail regions of the human β_2AR (Xiao and Shenoy, 2011). However, whether Iso and carvedilol target the same intracellular lysines for ubiquitination remains to be determined. Intriguingly, because carvedilol induces ubiquitination of the lysineless β_2AR mutant (0K-β_2AR) and because the viral counterparts of MARCH2 RING domain ligases have been shown to target noncanonical sites for ubiquitination (Cadwell and Coscoy, 2005), carvedilol-induced ubiquitination might involve cysteines and/or lysines or possibly other noncanonical sites (serines or threonines) within the β_2AR.

Although both Iso and carvedilol stimulate comparable β_2AR ubiquitination, the regulatory components and cellular consequences are distinct. Agonist-dependent ubiquitination requires β-arrestin2 (Shenoy et al., 2001, 2008), whereas carvedilol-induced ubiquitination does not; agonist-dependent ubiquitination is mediated by the HECT domain E3 ligase Nedd4 (Shenoy et al., 2008; Nabhan et al., 2010), whereas carvedilol-induced ubiquitination involves MARCH2 and not Nedd4; agonist-dependent internalization occurs in the absence of ubiquitination (Shenoy et al., 2001, 2008; Liang et al., 2008; Xiao and Shenoy, 2011), whereas carvedilol-induced internalization occurs only upon receptor ubiquitination by MARCH2; agonist-occupied β_2ARs can recycle and engage USP20 and USP33 activities, whereas carvedilol binding blocks recycling and binding of these USPs. These differences are likely because of the distinct receptor conformations induced by each ligand (Galandrin et al., 2007; Rosenbaum et al., 2009; DeWire and Violin, 2011), which leads to binding of specific protein partners to mediate distinct cellular consequences. Recently arrestin-like proteins have been shown to be important for mediating the trafficking of plasma membrane receptors and channels in yeast and mammalian systems (Lin et al., 2008; Nikko et al., 2008; Polo and Di Fiore, 2008; Nabhan et al., 2010; Becuwe et al., 2012). However, these proteins may not play an important role in carvedilol-induced β_2AR trafficking because they act mainly as protein partners for HECT domain E3 ligases and may not critically regulate the RING domain–containing MARCH family ligases.

Carvedilol, is currently defined as a β-arrestin–biased agonist because it can stimulate β-arrestin–dependent signaling while blocking G protein coupling (Wisler et al., 2007). Our findings

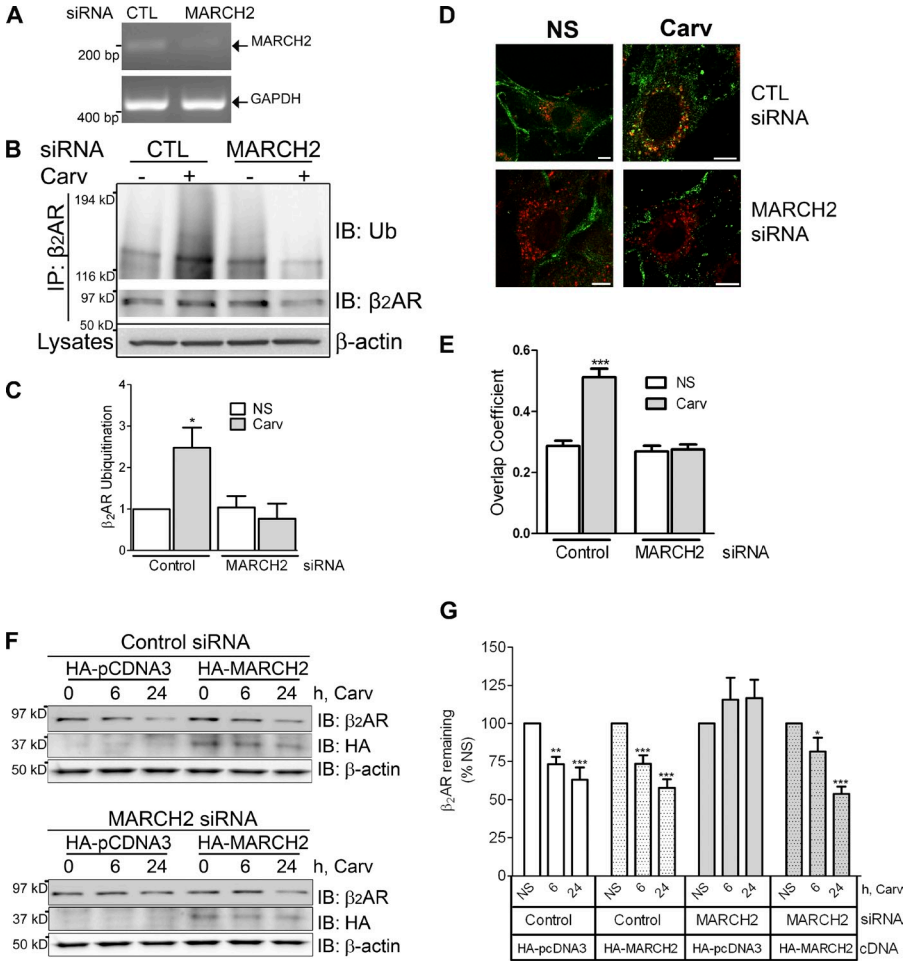

Figure 8. **MARCH2 expression is required for carvedilol-induced ubiquitination, lysosomal trafficking, and degradation of endogenous β_2ARs.** (A) Rat VSMCs were transfected with either control (CTL) or MARCH2 siRNA; total RNA was extracted 48 h later and subjected to RT-PCR for MARCH2 or GAPDH, as indicated. There was an 80 ± 5% knockdown of MARCH2 mRNA (n = 6). (B) Rat VSMCs transfected with CTL or MARCH2 siRNA were stimulated with carvedilol (1 h, 37°C), and β_2AR immunoprecipitates were probed with anti-Ub (top) or anti-β_2AR (bottom) IgG. (C) The ubiquitin signals were quantified, normalized to the β_2ARs in each IP sample, and plotted as bar graphs (mean ± SEM). Compared with NS: *, $P < 0.05$; n = 5. (D) Rat VSMCs transfected with control or MARCH2 siRNA were treated with carvedilol and stained with LysoTracker red and for the β_2AR (green; as described in Fig. 2). Bars, 10 µm. (E) Pearson's correlation coefficients were calculated for β_2AR and LysoTracker red colocalization for NS and carvedilol-challenged VSMCs and plotted as bar graphs (mean ± SEM). Compared with NS: ***, $P < 0.001$; n > 20 VSMCs for all conditions. (F) Rat VSMCs were transiently transfected with control or MARCH2 siRNA along with either vector (HA-pCDNA3) or HA-MARCH2. Cells were then stimulated with 1 µM carvedilol for indicated times in the presence of 20 µM cycloheximide, and then solubilized. VSMC lysates were immunoblotted serially with IgG specific for the β_2AR, HA, and β-actin. (G) The signals for β_2AR were quantified from eight independent experiments, normalized to β-actin levels, and plotted as mean ± SEM. As analyzed by two-way ANOVA, only carvedilol-treated MARCH2 siRNA + pCDNA3 samples were significantly different from the counterparts in all other groups. Statistical analysis per one-way ANOVA within each group is displayed. Compared with cognate NS VSMCs: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

show that this β-arrestin–biased agonist can lead to β-arrestin–independent pathways: recruitment of MARCH2 and β-arrestin–independent endocytosis of the β_2AR. Notably, both early endocytosis and postendocytic sorting effects induced by this biased agonist are dependent on MARCH2. This reveals yet another tier of complexity of receptor–ligand interaction, where a ligand can be biased toward a particular protein recruitment to engage a specific intracellular pathway: carvedilol recruits (a) β-arrestin transiently to stimulate MAPK signals and (b) MARCH2 to facilitate receptor endocytosis and lysosomal sorting in a ubiquitin-dependent manner.

MARCH2 is a member of a small family of RING-CH ligases consisting of eleven genes (Ohmura-Hoshino et al., 2006; Nathan and Lehner, 2009; Eyster et al., 2011). MARCH ligases are the cellular orthologues of the viral immunoevasion ligases K3 and K5 (Wang et al., 2008). Since their discovery in 2000, MARCH genes have been difficult to associate with relevant physiological roles (Nathan and Lehner, 2009). MARCH2 is a recently characterized E3 ubiquitin ligase that is ubiquitously expressed and is often associated with endosomal membranes. MARCH2 displays direct binding with syntaxin6 and has been shown to regulate Transferrin receptor trafficking (Nakamura et al., 2005). The PDZ ligand motif of MARCH2 participates in recognition and binding to the PDZ domain protein DLG1 (Cao et al., 2008). Whether MARCH2 is recruited by other PDZ domain proteins such as NHERF1 and 2 that regulate β_2AR trafficking (Ritter and Hall, 2009) remains to be determined.

Carvedilol is a third generation β blocker prescribed in the US specifically to treat heart failure and has been shown to improve left ventricular ejection fraction and reduce mortality in heart failure patients (Ruffolo and Feuerstein, 1997). It is a nonselective βAR antagonist that lacks intrinsic sympathomimetic activity and is also a blocker of α_1AR (Vanderhoff et al., 1998). However, studies have confirmed that blocking α_1ARs alone does not confer an advantage in CHF (Cohn et al., 1986) and may even increase the incidence of heart failure in hypertensive patients (Lasagna, 2000). Carvedilol has also been shown to reduce myocardial infarction and morbidity in animal models of coronary artery occlusion, where propranolol and other β blockers had no significant effect (Hamburger et al., 1991; Feuerstein et al., 1992).

The degradation profile of β_2AR that we have demonstrated in VSMCs could be relevant to the effects of carvedilol in human heart and vasculature. At therapeutic doses, the peak

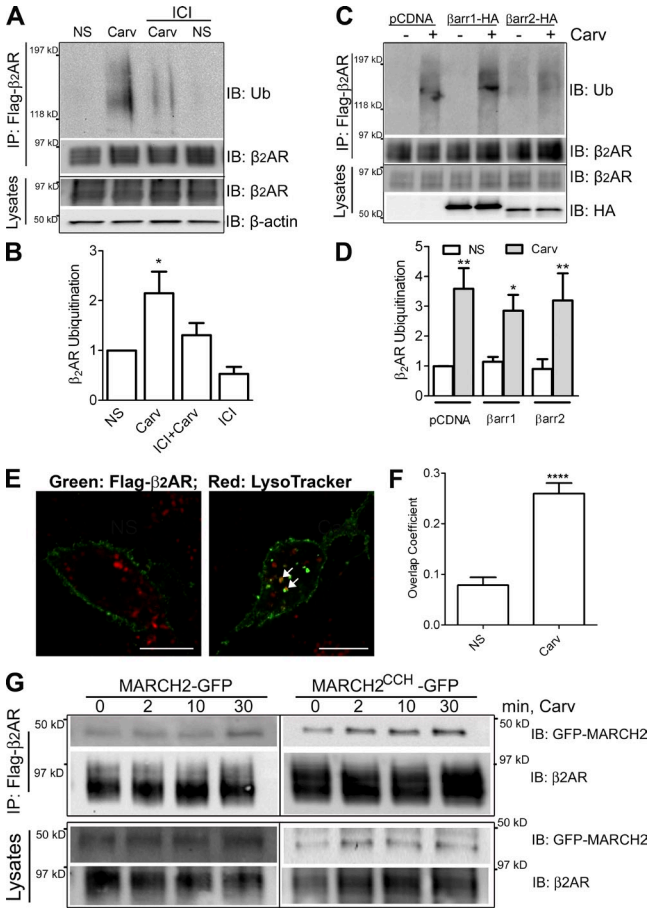

Figure 9. **Carvedilol induces β_2AR ubiquitination and trafficking and MARCH2 recruitment in β-arrestin1/2 KO MEFs.** (A) β-Arrestin1/2 double KO MEFs transfected with the Flag-β_2AR were pretreated with ±1 µM ICI 118,551 and then stimulated with ±10 µM carvedilol (1 h, 37°C). Flag immunoprecipitates were isolated and probed for ubiquitinated β_2ARs (top) and total β_2ARs (second panel). The bottom two panels show expression levels of β_2AR and β-actin as detected in solubilized extracts. (B) The ubiquitin signal in each IP was quantified and normalized to the β_2AR band; data from three experiments were plotted as bar graphs (mean ± SEM). *, $P < 0.05$, one-way ANOVA. (C) β-Arrestin1/2 KO MEFs were transfected with Flag-β_2AR along with vector, β-arrestin1–HA or β-arrestin2–HA plasmids. Flag immunoprecipitates were immunoblotted serially with anti-Ub and anti-β_2AR (H-20) IgG. MEF lysates were also probed for the β_2AR and β-arrestin1 and 2. (D) β_2AR ubiquitination was quantitated as in Fig. 1 D and plotted as the means ± SEM from four independent experiments. **, $P < 0.01$; *, $P < 0.5$ versus pCDNA3-NS. (E) Subcellular distribution of Flag-β_2AR as detected by surface labeling (as in Fig. 2) is shown in green and LysoTracker in red. After 1 h of carvedilol stimulation, receptors are seen in endocytic vesicles and partially colocalized with LysoTracker (arrows). Bars, 10 µm. (F) The bar graph (mean ± SEM) represents Pearson's correlation coefficients that were calculated for β_2AR and LysoTracker red colocalization for NS and carvedilol-stimulated conditions. ****, $P < 0.0001$; $n > 20$ cells for each condition. (G) β-Arrestin1/2 KO MEFs with stable expression of the Flag-β_2AR (600 fmol/mg of cellular protein) were transfected with either MARCH2-GFP or MARCH2^{CCH}-GFP stimulated with carvedilol for the indicated times. Flag immunoprecipates and MEF lysate blots were immunoblotted serially for MARCH2 and the β_2AR, as indicated. Shown are results from a single experiment, representative of three independent experiments.

plasma level of carvedilol is 100 ng/ml and the drug is mostly bound to plasma proteins. However, it accumulates in extravascular tissues, which could affect βAR cell surface expression and lead to prolonged blockade of βAR signaling. Other recent studies also suggest that in heart failure models, where β_1AR is down-regulated, β_2ARs are not and become redistributed and reprogrammed to carry out β_1AR-like functions, thus contributing to progression of heart failure (Nikolaev et al., 2010). This suggests that down-regulating the redistributed β_2ARs with carvedilol may be beneficial in heart failure patients. We believe that in this scenario MARCH2 activity might be critical in mediating the beneficial effects exerted by carvedilol in orchestrating cell surface levels of β_2AR and consequently its signaling.

Materials and methods

Cell lines

Human embryonic kidney cells (HEK-293) were purchased from American Type Culture Collections and maintained in minimal essential medium containing 10% FBS and 1% penicillin/streptomycin (P/S). HEK-293 cells stably transfected with Flag-β_2AR or Flag-β_2AR-YFP have been described previously (Shenoy et al., 2008; Berthouze et al., 2009). These cells were generated by transfecting early passage HEK-293 cells with 1 µg of plasmid DNA: Flag-β_2AR/pcDNA3 or Flag-β_2AR-mYFP/pcDNA3 and positive clones were selected against 1 mg/ml G418. Stable cells were further maintained by the addition of 400 µg/ml of G418 to the culture media. Aortic VSMCs were isolated from adult rats or mice using protocols reported previously and were maintained in DMEM supplemented with 10% FBS and 1% P/S (Kim et al., 2008b). To isolate these primary cells, aortas were dissected, washed with saline buffer, separated from the adventitia and endothelial cells, and then digested at 37°C for 1.5 h with 0.1% collagenase II, 15 U/ml elastase, and 0.1% soybean trypsin inhibitor mixed in PBS. Released smooth muscle cells were cultured in DMEM with 20% FBS and 1% P/S (Life Technologies) immediately after isolation and subsequent subcultures were maintained in DMEM supplemented with 10% FBS and 1% P/S. All experiments were repeated in cells isolated at least three independent times. Animals (C57/Bl6 mice or Sprague-Dawley rats) were purchased from vendors and housed in Duke University animal facilities. All animal procedures were performed according to protocols approved by Duke University Institutional Animal Care and Use Committee.

In early passage (<6) VSMCs the ratio of β_1/β_2 mRNA has been shown as 1:300 (Keys et al., 2005). Using radioligand binding (^{125}I-cyanopindolol ± ICI 181,551) we determined β_1AR levels to be negligible and β_2AR expression levels to mean 0.8–1.2 pmol/mg and 0.7–1.0 pmol/mg, respectively, in isolated rat and mouse VSMCs. In HEK-293 cells, the expression of endogenous β_2ARs is very low (~0.02 pmol/mg) and detection of ubiquitination of endogenous receptors was not achievable.

Plasmids and transfections

Human MARCH2/pCMV6-XL5 plasmid was purchased from OriGene. HA-MARCH2/pCDNA3 and MARCH2-pEGFPN1 were generated using standard cloning methods. To generate MARCH2^{CCH}, three amino acid mutations (cysteines 64 and 67 to serines and histidine 90 to glutamine) were introduced by QuikChange site-directed mutagenesis protocol. HEK-293 cells were transiently transfected by using Fugene6 reagent (Roche) according to the manufacturer's instructions. MEFs and VSMCs were transfected with either plasmids or siRNA oligos using Lipofectamine 2000. For siRNA rescue experiments, plasmids encoding human MARCH2 cDNA were transfected along with siRNA oligos that specifically targeted mouse or rat mRNA. For all siRNA transfections, 50–60% confluent monolayers were incubated with the transfection mixture in serum-free media for 24 h after when serum was replenished and 24 h later cells were used for various assays. This method routinely resulted in ~60% transfection efficiency of early passage VSMCs as assessed by transfection with a plasmid encoding GFP and examining GFP-positive cells.

Antibodies

Anti-β_2AR (M-20, H-20, and I3D6) antibodies were obtained from Santa Cruz Biotechnology, Inc. Anti-Nedd4 WW domain (EMD Millipore) was used to detect endogenous Nedd4 in HEK-293 cells. Anti-Nedd4 antibody (Cell Signaling Technology) was used for Western blots of rat VSMCs. Custom-generated anti-MARCH2 antibodies, anti-MAR2$_C$#41, anti-MAR2$_N$#51 (immunoblotting shown in Western blot panels), and anti-MAR2$_N$#384, have been reported previously (Nakamura et al., 2005). Anti-peptide antisera (anti-MAR2$_C$#41 and anti-MAR2$_N$#51) were generated in rabbits against synthetic peptides corresponding to residues 212–230 and to 42–61

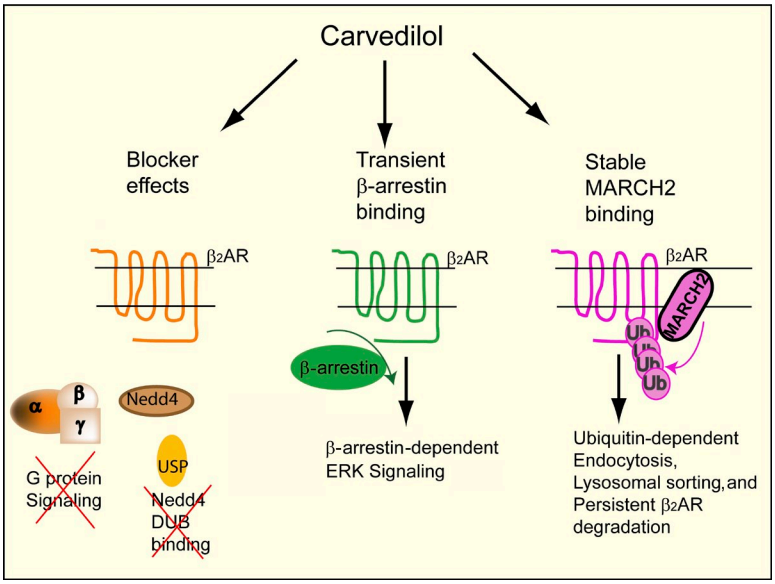

Figure 10. **Carvedilol induces distinct molecular effects.** Upon binding to the β_2AR, carvedilol prevents G protein coupling, recruits β-arrestin transiently to induce pERK signaling, and forms a stable complex with MARCH2 to promote ubiquitin-dependent endocytosis and lysosomal degradation. Carvedilol also blocks the association of the β_2AR with Nedd4, and USP20 and USP33, all of which are recruited to the β_2AR when it is activated by Iso.

of rat MARCH2, respectively. A rabbit polyclonal anti-MAR2$_N$#384 was raised against a GST-N fusion protein. Prokaryote expression vector for GST-MAR2$_N$ was constructed by cloning the fragment encoding residues 2–141 of rat MARCH2 into pGEX-4T. The commercial anti-MARCH2 antibodies that we have tested include the following: mouse polyclonal B01P (Abnova), rabbit polyclonal A01 (Abnova), goat polyclonal A-12 (Santa Cruz Biotechnology, Inc.), and polyclonal anti-MARCH2 (Abcam). Anti–β-actin antibody was obtained from Sigma-Aldrich. Anti-ubiquitin FK1 was purchased from Enzo Life Sciences and used to detect ubiquitination. For IPs from rat VSMCs, the blots were also assessed with a rabbit polyclonal ubiquitin antibody from Bethyl Laboratories, Inc. Secondary antibodies conjugated to HRP were obtained from GE Healthcare. Secondary antibodies conjugated to Alexa fluorophores and LysoTracker red were purchased from Invitrogen. Anti–β-arrestin antibodies and purified recombinant β_2AR protein were provided by R.J. Lefkowitz (Duke University, Durham, NC).

siRNA

Double-stranded siRNAs were chemically synthesized in deprotected and desalted form (Thermo Fisher Scientific). Sequences of siRNA oligonucleotides are as follow: control, non-targeting sequence, 5′-UUCUCCGAACGUGUCACGU-3′; Nedd4 (rat), 5′-AACUAUCAAAAAGUCUUUG-3′; RM-March2-1 (common to mouse and rat), 5′-GGAGAAAUGGCUGUCUUCC-3′; RM-March2-2 (common to mouse and rat), 5′-CAGCUACUGUGAGCUGUGU-3′; RM-March2-3 (common to mouse and rat), 5′-CUGGUCUCUUUCCGAUACC-3′; RM-β_2AR (common to mouse and rat), 5′-UGAUUGCAGUGGAUCGCUA-3′.

IP and immunoblotting

Rat or mouse VSMCs were serum deprived for 1 h and then stimulated with vehicle or carvedilol for 1 h. At the end of incubation, cells were harvested in a lysis buffer containing 50 mM Hepes, pH 7.5, 0.5% NP-40, 250 mM NaCl, 2 mM EDTA, and 10% (vol/vol) glycerol. All buffers were supplemented with protease inhibitors. Harvested cells were further solubilized by adding *n*-Dodecyl β-D-maltoside (1% final) and incubating on a rotator at 4°C for 1–2 h. After this, samples were centrifuged, soluble extracts were prepared, and protein concentrations were determined by Bradford analysis. Equal amounts of lysates were mixed with 2 µg anti-β_2AR M-20 or normal rabbit IgG along with protein A/G agarose beads and rotated overnight at 4°C. Nonspecific binding in the immunoprecipitate was eliminated by repeated washes with lysis buffer, and bound protein was eluted with sample buffer containing SDS. Samples were incubated at 37°C for 30 min before SDS-PAGE. The eluted proteins were separated on a gradient gel (4–20%; Invitrogen) and transferred to nitrocellulose membrane (0.2 µm; Bio-Rad Laboratories) for Western blotting. Anti-ubiquitin antibody was first used to probe the blots for ubiquitinated receptor bands, after which membranes were stripped (stripping solution; Thermo Fisher Scientific) and reacted with an anti-β_2AR antibody (M-20; Santa Cruz Biotechnology, Inc.) to detect receptors in the immunoprecipitates. Protein A–HRP was used instead of an anti–rabbit secondary antibody to minimize signals from IgG bands (Lal et al., 2005). Chemiluminescence detection was performed using SuperSignal West Pico or Femto reagent (Thermo Fisher Scientific). Signals were detected using a charge-coupled device camera (Chemidoc XRS; Bio-Rad laboratories) and quantified using ImageLab 3.0 software (Bio-Rad Laboratories). Analysis of Nedd4/β_2AR and MARCH2/β_2AR interactions were performed using Dithio-bis-maleimidoethane (DTME; Thermo Fisher Scientific) as described previously (Shenoy et al., 2007). Cells plated on poly-D-lysine–coated 100-mm dishes were stimulated at 37°C in PBS containing 10 mM Hepes, pH 7.5, with vehicle or agonist. Stimulations were terminated by the addition of DTME to a final concentration of 2 mM, and plates were rocked for 40 min at room temperature. Cells were washed three times with PBS/Hepes to remove unreacted DTME and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 1% NP-40, and 0.5% deoxycholate) and receptors were immunoprecipitated.

Immunostaining of endogenous β_2ARs in VSMCs

Early passage (<6) VSMCs were plated on collagen-coated glass-bottomed dishes (MatTek Corporation). When needed, the cells were transfected with siRNA using Lipofectamine 2000 as described in the previous section. Cells were washed once with DMEM containing 0.1% BSA and 10 mM Hepes (DMEM-BSA), pH 7.5, and covered with the mouse monoclonal anti-β_2AR antibody I3D6 in DMEM-BSA (dilution 1:100). LysoTracker red was added to the antibody solution (1:1,000 dilution) for simultaneous uptake of the dye. The antibody feeding was performed at room temperature for 1 h. After, the cells were stimulated with vehicle or carvedilol and returned to a 37°C incubator for desired times, and then, at the end of stimulation, the cells were washed with PBS and fixed with 5% formaldehyde for 30 min. Secondary antibody (anti–mouse IgG) conjugated to Alexa Fluor 488 was added and the cells were labeled at 4°C overnight. Immunostained cells (on the glass-bottom dish) covered with PBS buffer were imaged at room temperature with a confocal microscope (LSM 510 META; Carl Zeiss) using a Plan-Apochromat 100× NA 1.4 objective lens (Carl Zeiss). All confocal analyses were performed on samples from three to five independent experiments. In each experiment, several cells or groups of cells were analyzed. Image acquisition used the LSM 510 operating software and images were later exported as TIFF files. Further processing (resizing, addition of text, etc.) was performed using Adobe Photoshop software (CS2) and any change in brightness/contrast was applied to the entire image. Pearson's correlation coefficients for quantification of β_2AR-LysoTracker colocalization was performed in ≥20 cells from multiple independent experiments using ImageJ software (National Institutes of Health).

Internalization by ELISA

To quantify cell surface β_2ARs, cells were plated in either 24- or 96-well dishes. Cell surface receptors were prelabeled with the mouse monoclonal I3D6-β_2AR antibody (Santa Cruz Biotechnology, Inc.) diluted at 1:300 in serum-free media containing 10 mM Hepes, pH 7.4, and 0.01% BSA at 4°C for 1 h. Cells were then washed and exposed to either

carvedilol or Iso (both at 1 µM) for 1 h at 37°C. Subsequently, cells were washed with PBS and labeled with alkaline phosphatase–conjugated goat anti–mouse antibody for 1 h at 4°C. After this, unbound antibodies were removed by repeated washing with PBS and color development was induced by adding one-step *p*-nitrophenyl phosphate disodium salt (Thermo Fisher Scientific). After 10 min of development, the reaction was stopped by adding 2N NaOH. Absorbance was measured at 405 nm on a plate reader (Bio-Rad laboratories). Samples labeled with secondary antibody alone were used as background controls. The amount of cell surface receptors is presented as a percentage of cell surface receptors in unstimulated cells. For the experiments involving endocytosis inhibitors, either vehicle (DMSO), 100 µM MDC, or 80 µM dynasore were added 5 min before stimulation of the cells. For experiments involving recycling, ligands were removed by washing and adding fresh media (warmed at 37°C) containing 10 mM Hepes, pH 7.4, and 0.01% BSA and incubation at 37°C for 1 h.

Proteomics analysis

HEK-293 cells stably transfected with Flag-β_2AR were used for preparation of the β_2AR complexes. Cells were grown to ∼75% confluency and treated with buffer, 10 µM Iso or 10 µM carvedilol for 6 h before harvesting. The harvested cells were solubilized with Lysis buffer (50 mM Hepes, 0.5% NP-40, 250 mM NaCl, 10% Glycerol, and 2 mM EDTA). Next, *n*-Dodecyl β-D-maltoside (1% final) was added and samples were rotated at 4°C for 1–2 h. The solubilized β_2AR complexes were isolated with anti-Flag (M2) affinity agarose beads, eluted with sample buffer, and separated by SDS-PAGE (4–20% gradient gel; Invitrogen). Each sample lane on the SDS-PAGE gel was demarcated in to four to five sections, excised, chopped into small pieces, and subjected to in-gel trypsin digestion. In brief, the gel pieces were destained by 25 mM of ammonium bicarbonate in 50% acetonitrile. The samples were reduced by 2 mM dithiothreitol, alkylated by 10 mM iodoacetamide, and then subjected to trypsin (final concentration of 5 ng/µL) digestion at 37°C overnight. Tryptic peptides were subjected to LC-MS/MS analyses on an LTQ Orbitrap XL (Thermo Scientific) with a Finnigan Nanospray II electrospray ionization source. Tryptic peptides were injected onto a 75 µm × 150 mm BEH C18 column (particle size 1.7 µm; Waters) and separated using a nanoACQUITY Ultra Performance LC system (Waters; Xiao and Shenoy, 2011). The LTQ Orbitrap XL was operated in the data-dependent mode using the TOP10 strategy (Haas et al., 2006). Each scan cycle was initiated with a full MS scan of high mass accuracy (400–2,000 m/z; acquired in the Orbitrap XL at 6×10^4 resolution setting and automatic gain control target of 10^6). This was followed by MS/MS scans (automatic gain control target of 5,000; threshold 3,000) in the linear ion trap on the 10 most abundant precursor ions. Selected ions were dynamically excluded for 30 s. Singly charged ions were excluded from MS/MS analysis. MS/MS spectra were searched by using the Mascot (Matrix Sciences, Inc.) algorithm against a composite database containing the SwissProt *Homo sapiens* (human) protein sequences and their reverse sequences. Search parameters allowed two missed tryptic cleavages, a mass tolerance of ±10 ppm for precursor ion, a mass tolerance of ±0.02 D for product ion, a static modification of 57.02146 D (carboxyamidomethylation) on cysteine, and a dynamic modification of 15.99491 D (oxidation) on methionine.

β_2AR degradation

To detect a change in total receptor protein, VSMCs were stimulated with carvedilol for increasing times in the presence of 20 µM cycloheximide. At the end of incubation, cells were washed with PBS and harvested in 2× SDS sample buffer and briefly sonicated on ice (for 10–15 s; Marchese and Benovic, 2001). 30 µg of each sample were separated by SDS-PAGE and immunoblotted with the anti-β_2AR antibody M-20 (1:500 dilution). The same blots were stripped with a Western blot stripping solution (Thermo Fisher Scientific) and reprobed with anti–β-actin antibody to assess equal loading.

RT-PCR analyses

After transfection with siRNA, cells were harvested in 1 ml Ultraspec RNA, total RNA isolation reagent (Biotex Laboratories, Inc.), and prepared by chloroform extraction and isopropanol precipitation. To confirm the reduction of MARCH2 RNA levels by treatment of MARCH2-specific siRNA, RT-PCR was performed using 1–2 µg of total RNA with the one step RT-PCR kit (QIAGEN) using the manufacturer's protocol. The following primers were used: mouse and rat forward, 5′-GGAAAAGCGGCCCCGACCTC-3′; reverse, 5′-CAGCCTCCAGCCGGCTATGC-3′.

Statistical analyses

Experimental results shown are mean ± SEM for data averaged from at least three independent experiments. The *n* value shown in figure legends represents experiments done on independent occasions and in the case of primary cells, at least from three independent isolations. To determine significance, results were compared with control condition by means of *t* test (for two samples) or by analysis of variance (ANOVA) with Bonferroni post-test (for more than two samples). All statistical analyses were performed using Prism software (version 5; GraphPad Software). $P < 0.05$ at the 95% confidence level was considered significant.

Online supplemental material

Fig. S1 shows immunoblotting and immunostaining specificity and negative controls for the detection of endogenously expressed β_2ARs in VSMCs. Fig. S2 shows carvedilol-stimulated trafficking of YFP-tagged β_2ARs. Fig. S3 includes identification of novel regulators of β_2AR trafficking using proteomics. Fig. S4 shows subcellular distribution of MARCH2 and MARCH2^{CCH} ± carvedilol. Fig. S5 demonstrates that carvedilol-induced ubiquitination, internalization, and degradation of endogenous β_2ARs in mouse VSMCs are mediated by MARCH2. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201208192/DC1.

We thank Dr. R.J. Lefkowitz for insightful comments and for providing β-arrestin and β_2AR reagents. We also thank Drs. Arthur Moseley, Will Thompson, and Erik Soderblom for their input in the proteomics experiments; and Ms. Vidya Venkat for technical help.

We acknowledge support from the National Institutes of Health (HL 080525 to S.K. Shenoy, HL 77185 to N.J. Freedman, and HL 075443-Proteomics Core support to K.H. Xiao). S. Han was supported by the American Recovery and Reinvestment Act stimulus award (HL 080525-04S1).

Submitted: 31 August 2012
Accepted: 18 October 2012

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