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Differential Regulation of the Cell-Surface Targeting and Function of β- and α_1-Adrenergic Receptors by Rab1 GTPase in Cardiac Myocytes

Catalin M. Filipeanu, Fuguo Zhou, Erin K. Fugetta, and Guangyu Wu

Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center, New Orleans, Louisiana

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ABSTRACT

The molecular mechanism underlying the export from the endoplasmic reticulum (ER) to the cell surface and its role in the regulation of signaling of adrenergic receptors (ARs) remain largely unknown. In this report, we determined the role of Rab1, a Ras-like GTPase that coordinates protein transport specifically from the ER to the Golgi, in the cell surface targeting and function of endogenous β- and α_1-ARs in neonatal rat ventricular myocytes. Adenovirus-driven expression of Rab1 into myocytes selectively increased the cell-surface number of α_1-AR, but not β-AR, whereas the dominant-negative mutant Rab1N124I significantly reduced the cell-surface expression of β-AR and α_1-AR. Brefeldin A inhibited β-AR and α_1-AR export and antagonized the Rab1 effect on α_1-AR expression. Manipulation of Rab1 function similarly influenced the transport of α_{1A}- and α_{1B}-ARs as well as β_1- and β_2-ARs. Fluorescent microscopy analysis demonstrated that expression of Rab1N124I and Rab1 small interfering RNA induced a marked accumulation of GFP-tagged β_2-AR and α_{1B}-AR in the ER. Consistent with the effects on receptor cell-surface targeting, Rab1 selectively enhanced ERK1/2 activation and hypertrophic growth in response to the α_1-AR agonist phenylephrine but not to the β-AR agonist isoproterenol. Rab1N124I inhibited both agonist-mediated ERK1/2 activation and hypertrophic growth in neonatal myocytes. These results demonstrate that the cell-surface targeting and signaling of β- and α_1-ARs require Rab1 and are differentially modulated by augmentation of Rab1 function. Our data provide strong evidence implicating the ER-to-Golgi traffic as a site for selective manipulation of distinct AR function in cardiac myocytes.

β- and α_1-adrenergic receptors (ARs) play a critical role in the regulation of cardiac growth and function both at normal and diseased conditions (Post et al., 1999; Rockman et al., 2002). Three subtypes of β-ARs (β_1-AR, β_2-AR, and β_3-AR) and at least two subtypes of α_1-ARs (α_{1A}-AR and α_{1B}-AR) have been identified in the mammalian hearts. These receptors belong to the seven transmembrane spanning receptor superfamily coupled to heterotrimeric G proteins. β-ARs are coupled primarily to the stimulatory G protein Gs, whereas α_1-ARs are coupled to the G protein Gq (Zhong and Minneman, 1999; Zhu et al., 2001; Xiang and Kobilka, 2003). The precise function of ARs is determined by their intracellular trafficking and targeting, which are highly coordinated by many regulatory factors at distinct organelles. ARs are synthesized in the ER and then transported to the plasma membrane through the Golgi apparatus, where the receptors are post-translationally modified to attain mature status (Wu et al., 2003; Duvernay et al., 2005). Once at the plasma membrane, ARs may undergo internalization to the endosome upon stimulation by their ligands. Receptor internalization involves phosphorylation by at least two kinases, protein kinase A and G protein receptor kinases and subsequent binding of the phosphorylated receptors to arrestins, which serves as adaptor proteins recruiting components of the transport machinery to the clathrin-coated pits and initiating formation of the early endosome (Krupnick and Benovic 1998; Baillie et al., 2003). The internalized receptors in the early endosome may be sorted to the lysosome for degradation or to the recycling endosome for return to the plasma

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ABBREVIATIONS: AR, adrenergic receptor; ER, endoplasmic reticulum; BFA, brefeldin A; PE, phenylephrine; ISO, isoproterenol; ICI 118,551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; CEC, chloroethylclonidine; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; AT1R, angiotensin II type 1A receptor; ERK, extracellular signal-regulated kinase; CGP12177, 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one; siRNA, small interfering RNA; WT, wild type.

membrane (Hertel and Staehelin, 1983; Morrison et al., 1996). The balance of intracellular trafficking (i.e., export, endocytosis, recycling, and degradation) dictates the level of receptor at the plasma membrane and, in turn, will influence the magnitude of the cellular response to a given signal.

Compared with the extensive studies on the events of the endocytic pathway, the molecular mechanisms underlying the transport processes of ARs from the ER through the Golgi to the cell surface in cardiac myocytes and its role in the regulation of receptor function and in the development of cardiac disease remain largely unknown. Several studies have demonstrated that homo- and heterodimerization of ARs may be required for their export from the ER and subsequent transport to the cell surface (Xu et al., 2003; Salahpour et al., 2004; Zhou et al., 2006). For example, heterodimerization of α_{1B}-AR or β_2-AR with α_{1D}-AR enhances the cell-surface expression of α_{1D}-AR (Hague et al., 2004; Uberti et al., 2005). In addition, N-linked glycosylation of the receptors may also play important roles in targeting receptors to the cell surface as well as other specific transport pathways (Duvernay et al., 2005).

Rab proteins are Ras-like small GTPases that coordinate protein transport in almost every discrete step of the secretory and endocytic pathways (Plutner et al., 1991; Martinez and Goud, 1998; Takai et al., 2001). To date, 63 Rab GTPases have been identified in mammalian cells each with unique subcellular localization and mediating protein transport at specific steps (Takai et al., 2001). For example, Rab5 specifically mediates the transport of G protein-coupled receptors from the plasma membrane to the early endosome and Rab4 is involved in the recycling of internalized receptors from the endosome to the plasma membrane (Takai et al., 2001; Seachrist and Ferguson, 2003). Rab1 is localized in the ER and Golgi and exclusively regulates antegrade protein transport specifically from the ER to the Golgi and between the Golgi compartments (Plutner et al., 1991; Tisdale et al., 1992; Allan et al., 2000). We previously demonstrated that β_2-AR transport from the ER through the Golgi to the cell surface in HEK293T cells is dependent on Rab1, whereas α_{2B}-AR transport to the cell surface is independent of Rab1 (Wu et al., 2003). In this report, we determined the role of Rab1 in the cell surface targeting and signaling of distinct endogenous AR subtypes in cardiomyocytes. Our results demonstrate that cell surface expression and function of β-AR and α_1-AR are similarly attenuated by inhibiting Rab1 function but are differentially augmented by enhancing Rab1 function. These data indicate that the function of distinct ARs can be selectively modulated through manipulating their export trafficking in the early secretary pathway in cardiac myocytes.

Materials and Methods

Materials. [7-Methoxy-^{3}H]prazosin (specific activity, 70 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences. [^{3}H]CGP12177 (specific activity, 51 Ci/mmol) and [^{3}H]leucine (specific activity, 173 Ci/mmol) were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Brefeldin A (BFA), phenylephrine (PE), isoproterenol (ISO), atenolol, ICI 118,551, niguldipine, chloroethylclonidine (CEC), and anti-FLAG M2 monoclonal antibody were obtained from Sigma (St. Louis, MO). Antibodies against phospho-ERK1/2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and antibodies against ERK1/2 from Cell Signaling Technology Inc. (Beverly, MA). pDsRed2-ER, an ER marker, was from BD Biosciences (Palo Alto, CA). Rab1 was cloned from a mouse cardiac cDNA library (Wu et al., 2001). Human β_2-AR tagged with green fluorescent protein (GFP) at its carboxyl terminus was generated as described previously (Wu et al., 2003). Human GFP-tagged α_{1B}-AR was a kind gift of Dr. Kenneth P. Minneman (Emory University School of Medicine, Atlanta, GA) (Hague et al., 2004).

Isolation, Culture and Adenoviral Infection of Neonatal Rat Ventricular Myocytes. Neonatal ventricular myocytes were isolated from the hearts of 1- to 2-day-old Sprague-Dawley rats as described previously (Filipeanu et al., 2004; Li et al., 2005). The dominant-negative mutant Rab1N124I (a guanine nucleotide binding-deficient) was generated using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA). Adenoviruses expressing Rab1 and Rab1N124I tagged with the FLAG epitope at their amino termini were generated as described previously (Filipeanu et al., 2004). Isolated neonatal cardiac myocytes were cultured in DMEM and infected with control parent adenovirus or adenovirus expressing Rab1 or its dominant-negative mutant Rab1N124I at a multiplicity of infection (m.o.i.) of 20. After 48 h of infection, expression of Rab1 was determined by Western blotting using a FLAG high-affinity monoclonal antibody. Fluorescent microscopic analyses after immunostaining with anti-FLAG antibodies revealed that greater than 95% of the cardiomyocytes were infected (Filipeanu et al., 2004). To determine whether adenoviral expression of Rab1 could induce cell death, cardiac myocytes viability was measured using Calcein-AM retention and de-esterification as described previously (Yakovlev et al., 2000). The data indicate that adenoviral expression of wild-type Rab1 or Rab1N124I in neonatal cardiomyocytes did not significantly influence cell viability (wild-type Rab1-infected cells, 86 ± 7%; Rab1N124I-infected cells, 93 ± 2% relative to control virus-infected cells; $n = 4$, $p > 0.05$).

Measurement of Cell Surface Receptors. Cell-surface expression of β-AR and α_1-AR in neonatal cardiomyocytes was measured by intact cell ligand binding as described previously (McLean et al., 1999; Ricci et al., 1999; Calls et al., 2000) with modifications. Myocytes were cultured on 12-well plates at a density of 5×10^5 cells/well and infected for 48 h. The myocytes were then incubated with the ligand [^{3}H]CGP12177 at a concentration of 20 nM for 2 h or with [7-methoxy-^{3}H]prazosin at a concentration of 10 nM for 90 min at room temperature. To measure expression of individual AR subtypes, myocytes were preincubated for 30 min with the AR subtype-selective antagonists: ICI 118,551 (β_2-AR), atenolol (β_1-AR), niguldipine (α_{1A}-AR), or CEC (α_{1B}-AR) (10 μM). Nonspecific binding was determined in the presence of alprenolol (β-AR) or phentolamine (α-AR) (20 μM) and accounted for less than 10% of the total binding. The cells were washed twice with ice-cold phosphate-buffered saline and digested with 1 ml of 1 M NaOH. All ligand binding assays were performed in triplicate. The radioactivity was counted by liquid scintillation spectrometry.

Measurement of ERK1/2 Activation. Activation of ERK1/2 was measured as described previously (Wu et al., 2003; Filipeanu et al., 2004). Myocytes were cultured on six-well plates at a density of 1×10^6 cells/well and infected for 48 h. Myocytes were stimulated with PE (10 μM) or ISO (10 μM) for 8 min with or without pretreatment with the AR antagonists ICI 115,881 or atenolol (100 nM) for 30 min. The reaction was terminated by the addition of 600 μl of 1× SDS gel loading buffer. After solubilizing the cells, 30 μl of total cell lysates was separated by 10% SDS-polyacrylamide gel electrophoresis. ERK1/2 activation was determined by immunoblotting to measure their phosphorylation with phospho-specific antibodies. The membranes were stripped and reprobed with anti-ERK1/2 antibodies to determine the total amount of kinases and to confirm equal loading of proteins. The signal was detected using ECL Plus (PerkinElmer Life and Analytical Sciences) and a Fuji Film luminescent image analyzer (LAS-1000 Plus) and quantitated using the Image Gauge program (version 3.4).

[^{3}H]Leucine Incorporation. Protein synthesis rate was determined as described previously (Thaik et al., 1995; van Kesteren et

al., 1997). In brief, neonatal cardiomyocytes were plated in 12-well dishes at a density of 5×10^5/well in DMEM supplemented with 10% fetal bovine serum. After infection with the desired construct, the myocytes were made quiescent by incubation in DMEM without fetal bovine serum for 48 h. The cardiomyocytes were then incubated with [^{3}H]leucine (1 μCi) for 24 h at 37°C in the presence or absence of the AR agonists ISO (10 μM) or PE (10 μM) with or without ICI 115,881 or atenolol. The reaction was terminated by aspirating the medium. The cardiomyocytes were washed twice with 1 ml of 5% trichloroacetic acid followed by an extraction with 1 ml of 5% trichloroacetic acid for 1 h in ice to remove nonincorporated [^{3}H]leucine. The cells were digested with 1 ml of 1 M NaOH for 6 h. The lysate was transferred to scintillation vials, neutralized with 1 ml of 1 M HCl, and counted by liquid scintillation spectrometry in 5 ml of Ecoscint A scintillation solution. Because Rab1 influences protein synthesis (Filipeanu et al., 2004), the effect of Rab1 on AR agonist-stimulated protein synthesis was calculated using the following formula: {[^{3}H]leucine incorporation in presence of agonist and Rab1] − [[^{3}H]leucine incorporation in presence of Rab1]}/{[^{3}H]leucine incorporation in presence of agonist and control adenovirus] − [[^{3}H]leucine incorporation in presence of control adenovirus]}.

Fluorescent Microscopy. Cardiomyocytes were grown on coverslips in six-well plates and infected with control, Rab1 or Rab1N124I adenoviruses as described above. After 10 h of infection, the medium was removed and the myocytes were transiently transfected using LipofectAMINE 2000 reagent (Invitrogen) as described previously (Wu et al., 2003; Filipeanu et al., 2004). One microgram of GFP-tagged AR with or without 1 μg of pDsRed2-ER construct were diluted into 125 μl of serum-free Opti-MEM in a tube. In another tube, 5 μl of LipofectAMINE was diluted into 125 μl of serum-free Opti-MEM. Five minutes later both solutions were mixed and incubated for another 20 min. The transfection mixture was added to culture dishes containing 0.8 ml of fresh Dulbecco's modified Eagle's medium and 10% fetal bovine serum without antibiotics. After transfection 36 to 48 h, the myocytes were fixed with a mixture of 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline for 15 min. The coverslips were mounted, and fluorescence was detected with a Leica DMRA2 epifluorescence microscope (Duvernay et al., 2004; Filipeanu et al., 2004). Images were deconvoluted using SlideBook software and the nearest-neighbors deconvolution algorithm (Intelligent Imaging Innovations, Denver, CO) as described previously (Filipeanu et al., 2004). Based on the GFP signal, approximately 5% myocytes were transfected by this plasmid transfection protocol.

Double-Stranded Small Interfering RNA. siRNA targeting the sequence at positions 136 to 156 of human Rab1 and a control nonsilencing siRNA were purchased from QIAGEN Inc. (Valencia, CA). Control and Rab1 siRNA were delivered into neonatal cardiomyocytes using LipofectAMINE 2000 reagent as described previously (Wu et al., 2003). In brief, 8 μl of LipofectAMINE 2000 and 6 μl of 20 μM siRNA were added separately to 100 μl of Opti-MEM. After incubation for 5 min, both solutions were mixed for 20 min. The transfection mixture was then added to the adenovirus-infected cardiac myocytes. The cells were incubated with the transfection mixture for 8 h and then medium was changed to standard culture medium. After 36 to 48 h, the cells were processed for fluorescence microscopy as described above.

Statistical Analysis. Differences were evaluated using Student's t test, and $p < 0.05$ was considered as statistically significant. Data are expressed as the mean ± S.E.

Results

Differential Regulation by Rab1 of β-AR and α_1-AR Expression at the Cell Surface in Cardiomyocytes. To determine whether Rab1 modulated export trafficking of endogenous ARs, we first determined the effect of transient expression of Rab1 and its dominant-negative mutant Rab1N124I on the cell-surface expression of β-AR and α_1-AR in primary cultures of neonatal rat ventricular myocytes. Myocytes were infected with control, Rab1, or dominant-negative mutant, Rab1N124I, adenoviruses (Fig. 1A), and the cell-surface expression of β-AR and α_1-AR was quantitated by ligand binding in intact myocytes using [^{3}H]CGP12177 and [^{3}H]prazosin, respectively. Cell surface expression of total β-AR and total α_1-AR was significantly attenuated by 60% and 58%, respectively, in cardiomyocytes infected with Rab1N124I adenovirus compared with cells infected with control adenovirus (Fig. 1B). It is noteworthy that, in contrast to Rab1N124I, adenoviral expression of Rab1 produced different effects on the cell surface expression of β-AR and α_1-AR. Whereas cell-surface expression of β-AR was not altered by Rab1, the cell-surface expression of α_1-AR was significantly augmented by 52% in cardiomyocytes infected with the Rab1 adenovirus compared with cells infected with control adenovirus (Fig. 1B). These data indicate that the levels of β-AR and α_1-AR expression at the cell surface depend on the normal Rab1 function and that augmentation of Rab1 function by overexpressing wild-type Rab1 may selectively facilitate the cell-surface targeting of endogenous α_1-AR in neonatal cardiomyocytes.

Rab1 GTPase regulates protein transport exclusively from the ER to the Golgi. To further determine the role of ER-to-Golgi transport in the cell-surface targeting of ARs, we de-

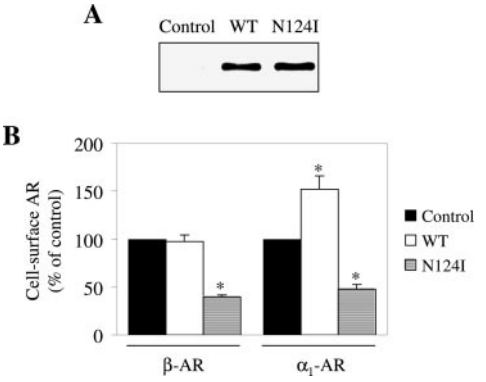

Fig. 1. Effect of adenovirus-mediated expression of Rab1 on the cell-surface number of β- and α_1-AR in neonatal rat ventricular myocytes. A, Western blot analysis of expression of wild-type Rab1 (WT) and its dominant-negative mutant Rab1N124I in adenovirus-infected myocytes. Neonatal myocytes were infected with empty adenoviral vector (Control) or recombinant FLAG-Rab1 adenovirus for 2 days at an m.o.i. of 20. Fifty micrograms of whole cardiomyocyte lysate was separated by 12% SDS-polyacrylamide gel electrophoresis, and FLAG-Rab1 expression was detected by immunoblotting with anti-FLAG antibody M2. The immunoblot is representative of results obtained in three different experiments. B, quantitation of cell surface number of β- and α_1-AR by intact cell ligand binding. Cardiomyocytes were cultured and infected with control, Rab1WT, or Rab1N124I adenoviruses for 2 days. The cell surface expression of β- and α_1-AR was determined by binding to ligands [^{3}H]CGP12177 and [^{3}H]prazosin, respectively, as described under *Materials and Methods*. Nonspecific binding of β- and α_1-AR was obtained in the presence of 20 μM alprenolol and 20 μM phentolamine, respectively, and subtracted from the values presented. The mean values of specific [^{3}H]CGP12177 binding were 5827 ± 421, 5654 ± 422, and 2323 ± 122 cpm (n = 3 each in duplicate) from the cardiomyocytes infected with control, Rab1WT, or Rab1N124I adenovirus, respectively. The mean values of specific [^{3}H]prazosin binding were 3065 ± 166, 4655 ± 436, and 1287 ± 141 cpm (n = 3 each in triplicate) from the cardiomyocytes infected with control, Rab1WT, or Rab1N124I adenovirus, respectively. The data shown are the percentage of the mean value obtained from the cardiomyocytes infected with control adenovirus and are presented as the means ± S.E. *, $p <$ 0.05 versus cardiomyocytes infected with control adenovirus.

termined whether BFA treatment could also modulate cell-surface expression of β-AR and α_1-AR. BFA is a fungal metabolite that disrupts the structures of the Golgi and blocks protein transport from the ER to the Golgi (Klausner et al., 1992; Yoo et al., 2002). Similar to the effect induced by adenoviral expression of Rab1N124I, BFA treatment reduced cell-surface numbers of β-AR and α_1-AR (Fig. 2A). Because expression of wild-type Rab1 selectively increased the cell-surface expression of α_1-AR, we determined whether BFA treatment could antagonize the effect of Rab1 on α_1-AR transport. Rab1-mediated increase in α_1-AR expression at the cell surface was significantly blocked by BFA treatment (Fig. 2B). Furthermore, α_1-AR expression at the cell surface was attenuated by BFA treatment in myocytes infected with Rab1N124. These data further indicate that the ER-to-Golgi transport plays an important role in the cell-surface targeting of β-AR and α_1-AR.

Regulation by Rab1 of Cell-Surface Expression of Individual AR Subtypes in Cardiomyocytes. As β-AR and α_1-AR each has multiple subtypes in cardiomyocytes, we sought to define β-AR and α_1-AR subtypes, whose transport from the ER to the cell surface is regulated by Rab1. Cell surface expression of β_1-AR, β_2-AR, α_{1A}-AR, and α_{1B}-AR, predominant β-AR and α_1-AR subtypes in cardiomyocytes, was determined by ligand binding in the presence of the AR subtype-selective antagonists atenolol, ICI 118,551, niguldipine, and CEC, respectively. Cell-surface expression of β_1-AR and β_2-AR was markedly inhibited by adenovirus-mediated expression of Rab1N124I but was not altered by wild-type Rab1 (Fig. 3A). Similar to β_1-AR and β_2-AR, cell-surface expression of α_{1A}-AR and α_{1B}-AR was also inhibited by Rab1N124I (Fig. 3). In contrast to β_1-AR and β_2-AR, cell surface expression of α_{1A}-AR and α_{1B}-AR was increased by adenoviral expression of Rab1 (Fig. 3B). These data are consistent with the effects of Rab1 on total β-AR and total α_1-AR expression at the cell surface and indicate that Rab1 regulation of export trafficking from the ER to the cell surface is indistinguishable between β_1-AR and β_2-AR and between α_{1A}-AR and α_{1B}-AR.

We then determined the effect of Rab1 on the subcellular localization of the ARs. To this end, GFP-tagged β_2-AR and α_{1B}-AR were transiently transfected into neonatal cardiomyocytes after infection with control or Rab1N124I adenoviruses. The subcellular distribution of the GFP-tagged receptors at steady state was revealed by fluorescent microscopy. As anticipated, β_2-AR-GFP and α_{1B}-AR-GFP were mainly localized at the cell surface in myocytes infected with control adenovirus. In contrast, β_2-AR-GFP and α_{1B}-AR-GFP were accumulated in the perinuclear regions and unable to transport to the cell surface in myocytes infected with Rab1N124I (Fig. 4A). These receptors were strongly colocalized with the ER marker pDsRed2 (Fig. 4B), consistent with the Rab1 function in regulating protein transport form the ER to the Golgi.

In the second series of experiment, we determined the effect of siRNA-mediated depletion of Rab1 on the subcellular localization of β_2-AR and α_{1B}-AR. Our previous data have

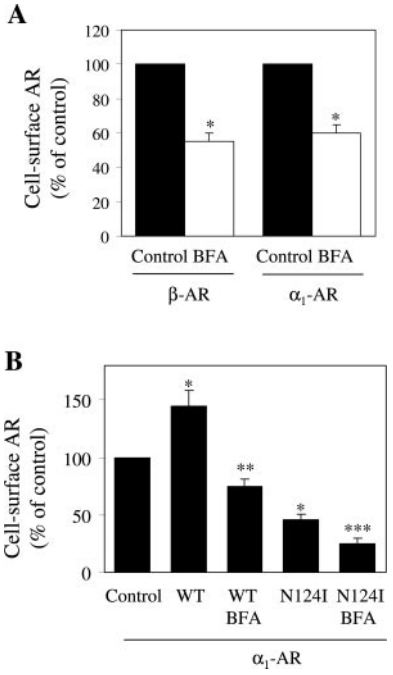

Fig. 2. Effect of BFA treatment on the cell-surface expression of β- and α_1-AR in cardiomyocytes. A, cardiomyocytes were cultured and incubated with ethanol (Control) or BFA at a concentration of 5 μg/ml for 8 h. The cell surface expression of β- and α_1-AR was determined as described in the legend of Fig. 1. The data shown are the percentage of the mean value obtained from the cardiomyocytes treated with ethanol and are presented as the means ± S.E. (n = 3 each in triplicate). *, $p < 0.05$ versus cardiomyocytes treated with ethanol. B, cardiomyocytes were cultured and infected with control, Rab1WT, or Rab1N124I adenoviruses for 2 days before the treatment with BFA. The data shown are the percentage of the mean value obtained from the cardiomyocytes infected with control adenovirus and are presented as the means ± S.E. (n = 3 each in triplicate) *, $p < 0.05$ versus cardiomyocytes infected control adenovirus; *, **, and ***, $p < 0.05$ versus cardiomyocytes infected with control, Rab1WT, and Rab1N124I adenovirus, respectively.

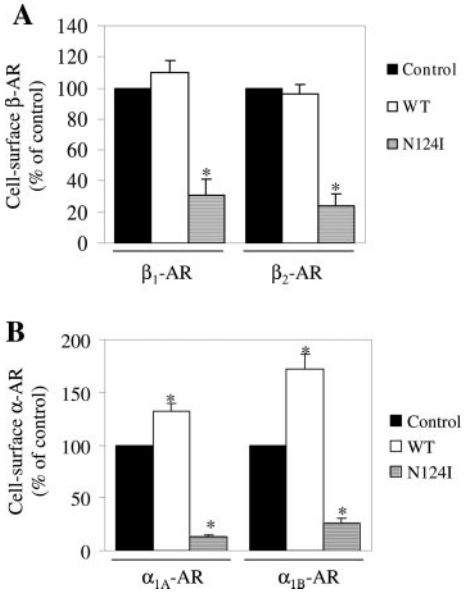

Fig. 3. Effect of Rab1 on cell-surface number of the β-AR subtypes β_1-AR and β_2-AR (A) and α_1-AR subtypes α_{1A}-AR and α_{1B}-AR (B) in neonatal cardiomyocytes. A, cardiomyocytes were cultured and infected with control, Rab1WT, or Rab1N124I adenoviruses for 2 days. The cell surface expression of β_1-AR and β_2-AR was measured by [^{3}H]CGP12177 binding in the presence of atenolol and ICI 118,551 (10 μM), respectively. B, the cell surface expression of α_{1A}-AR and α_{1B}-AR was measured by [^{3}H]prazosin binding in the presence of niguldipine and CEC, respectively. The data shown are the percentage of the mean value obtained from the cardiomyocytes infected with control adenovirus and are presented as the means ± S.E. (n = 3 each in triplicate) *, $p < 0.05$ versus cardiomyocytes infected with control adenovirus.

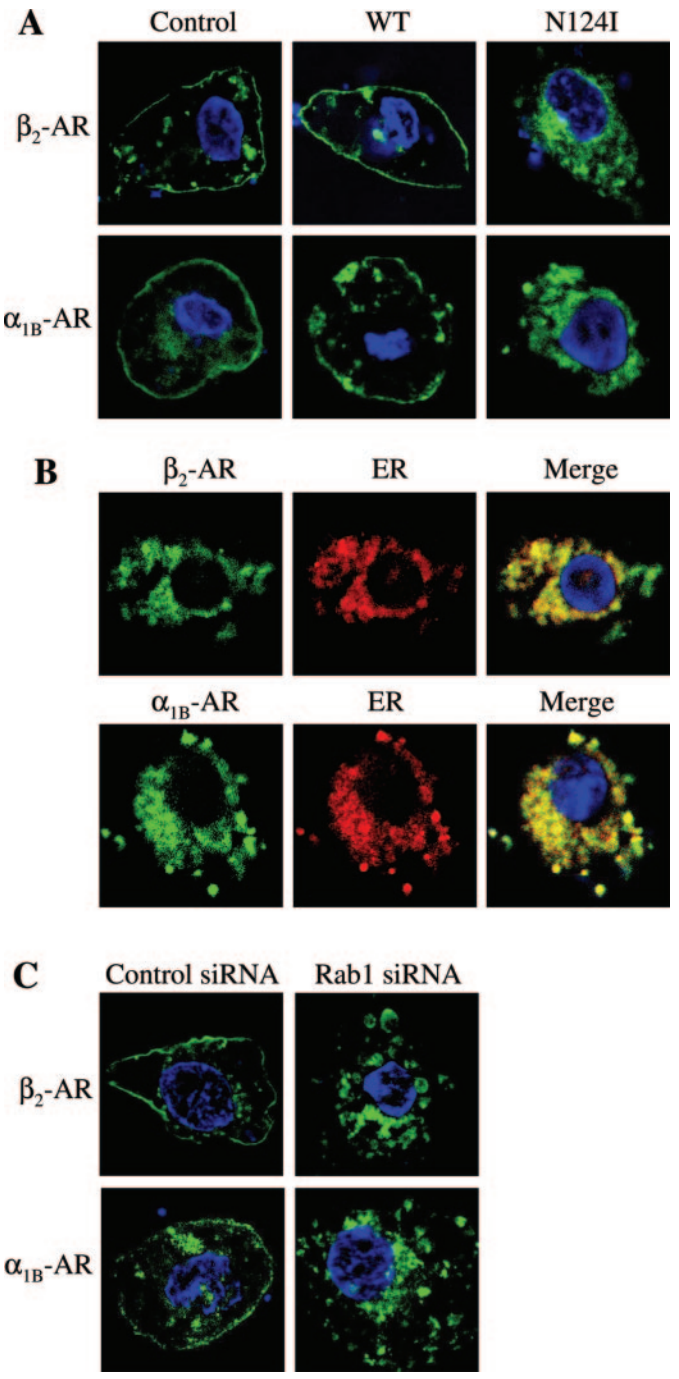

Fig. 4. Effect of Rab1 on the subcellular localization of β_2-AR and α_{1B}-AR in neonatal cardiomyocytes. A, the effect of adenoviral expression of Rab1 on the subcellular distribution of β_2-AR and α_{1B}-AR. Cardiomyocytes were grown on coverslips and infected with control, Rab1WT, or Rab1N124I adenoviruses and then transiently transfected with 1 μg of GFP-tagged β_2-AR and α_{1B}-AR using LipofectAMINE 2000. The subcellular distribution of the receptor was revealed by fluorescence microscopy as described under *Materials and Methods*. B, colocalization of receptors with the ER marker. Cardiomyocytes were grown on coverslips and infected with Rab1N124I adenoviruses. The cells were then transfected with GFP-tagged β_2-AR or α_{1B}-AR together with the ER marker pDsRed2-ER. C, the effect of transient expression of Rab1 siRNA on the subcellular distribution of β_2-AR and α_{1B}-AR. Cardiomyocytes were transiently transfected with GFP-tagged β_2-AR (top) or α_{1B}-AR (bottom) together with control siRNA (left) or Rab1 siRNA (right). The data are representative images of at least three independent experiments. Blue, DNA staining by 4,6-diamidino-2-phenylindole (nuclear); green, GFP-tagged receptor; red, the ER marker pDsRed2-ER; yellow, colocalization of GFP-tagged receptors with the ER marker.

demonstrated that transient transfection of Rab1 siRNA selectively reduced the expression of endogenous Rab1 (Wu et al., 2003). Consistent with the Rab1N124I effect, transfection of Rab1 siRNA induced an accumulation of both β_2-AR and α_{1B}-AR in the perinuclear regions compared with that in myocytes transfected with control siRNA (Fig. 4C). These data strongly indicate that normal Rab1 level is required for the transport of β_2-AR and α_{1B}-AR to the cell surface.

Modulation of β-AR and α_1-AR Signaling by Rab1 in Neonatal Cardiomyocytes. To determine whether Rab1 is capable of regulating AR signaling through modifying export trafficking of the receptors, we determined the effect of Rab1 on AR-mediated ERK1/2 activation. Neonatal myocytes infected with control, Rab1, and Rab1N124I adenovirus were stimulated with the nonselective β-AR agonist ISO in the absence or presence of atenolol or ICI 118,551. ERK1/2 activation in response to stimulation with the AR agonists was evaluated by measuring their phosphorylation. ISO-mediated ERK1/2 activation in the absence or presence of the antagonists was similarly inhibited in cardiomyocytes infected with Rab1N124I but was not altered in cardiomyocytes infected with Rab1 compared with control adenovirus-infected myocytes (Fig. 5). In contrast, PE-mediated ERK1/2 activation was attenuated by Rab1N124I and augmented by Rab1 (Fig. 5), suggesting that augmentation of Rab1 function by overexpressing Rab1 may selectively regulate AR signaling. These data are consistent with Rab1 effects on the cell surface expression of the receptors and indicate that Rab1 modulates not only AR traffic but also their signal transduction.

Effect of Rab1 on β-AR - and α_1-AR-Mediated Hypertrophic Response in Neonatal Cardiomyocytes. Our preceding data indicate that adenovirus-mediated expression of Rab1 selectively regulates the cell surface expression and signaling of ARs. We then determined whether manipulation of Rab1 function could influence hypertrophic growth by measuring total protein synthesis and sarcomeric organization in response to the agonists ISO and PE in cardiomyocytes. PE effects on protein synthesis were significantly increased by 54% in cardiomyocytes expressing Rab1 compared with cardiomyocytes infected with control adenovirus (Fig. 6A). In contrast, Rab1 had no influence on the protein synthesis in response to stimulation with ISO in the absence or presence of β_1- and β_2-AR antagonists (Fig. 6A). Total protein synthesis obtained from myocytes infected with parent adenoviral vector were close to that obtained from noninfected myocytes (data not shown), suggesting that influence of Rab1 infection on the protein synthesis could not be attributed to nonspecific effects of adenoviral infection.

In contrast to Rab1, expression of the dominant-negative mutant Rab1N124I significantly attenuated total protein synthesis in response to both agonists ISO and PE (Fig. 6A). Increases in total protein synthesis in response to stimulation with ISO and PE were markedly attenuated in cardiomyocytes infected with Rab1N124I compared with cardiomyocytes infected with control adenovirus (Fig. 6A). Furthermore, Rab1N124I infection inhibited sarcomeric organization in response to stimulation with both ISO and PE (Fig. 6B). These data indicate that the reduction of Rab1 function prevents AR-mediated cardiomyocyte hypertrophic growth, consistent with a decrease in cell-surface expression

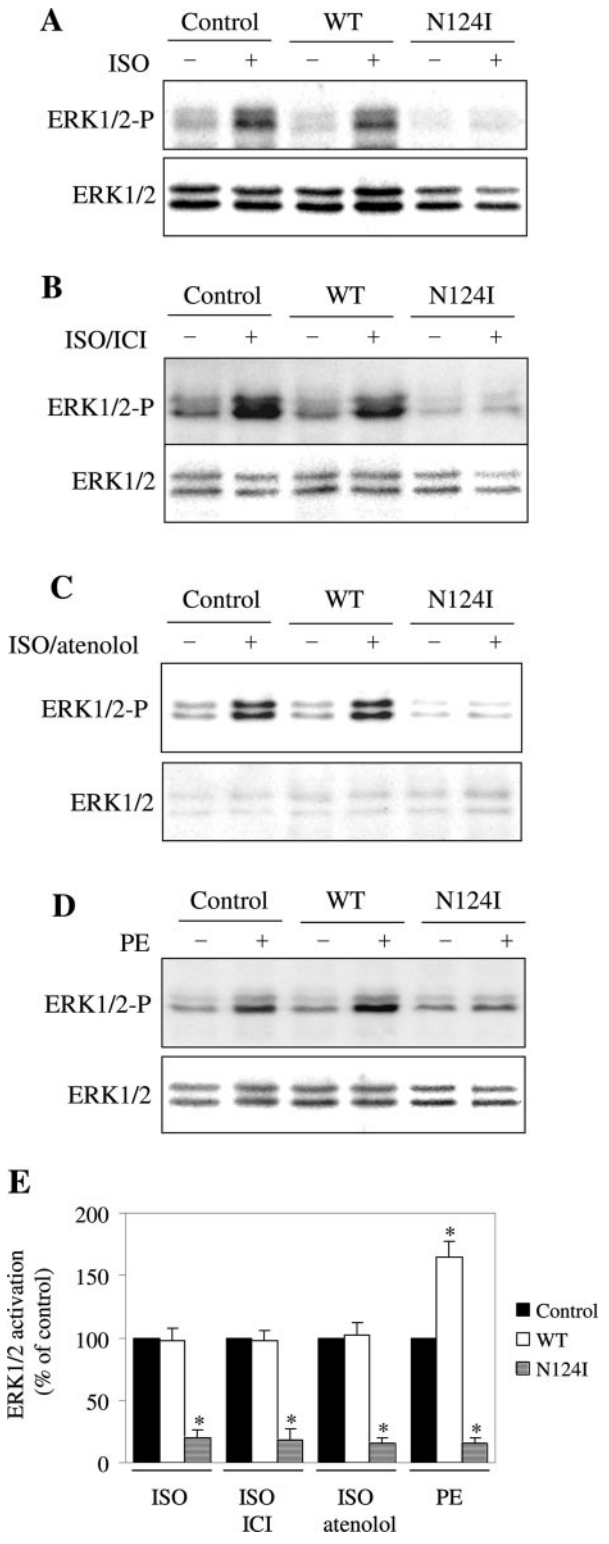

Fig. 5. Effect of Rab1 on β-AR- and α_1-AR-mediated ERK1/2 activation in neonatal cardiomyocytes. Cardiomyocytes cultured in six-well dishes were infected with control, Rab1WT, or Rab1N124I adenoviruses at an m.o.i. of 20 for 2 days. The cardiomyocytes were then stimulated at 37°C with ISO (10 μM) (A), ISO plus ICI 118,551 (10 μM) (B), ISO plus atenolol (10 μM) (C), or PE (10 μM) (D). The activation of ERK1/2 was determined by Western blot analysis using phospho-specific ERK1/2 (ERK1/2-P) antibodies. Representative blots of ERK1/2 (top) and total ERK1/2 expression (bottom) are shown. E, quantitative data expressed as the percentage of the mean value obtained from the cardiomyocytes infected with control adenovirus and presented as the means ± S.E. of at least three individual experiments. *, $p < 0.05$ versus cardiomyocytes infected with control adenovirus.

and signaling of ARs induced by the dominant-negative mutant Rab1N124I in cardiomyocytes.

Discussion

The most significant finding in this report is that augmentation of Rab1 function by adenovirus-mediated expression of wild-type Rab1 selectively influenced export trafficking of endogenous β- and α_1-ARs in neonatal ventricular myocytes. Overexpression of Rab1 significantly increased the total cell-surface number of α_1-AR as measured by intact cell ligand binding. In contrast, Rab1 expression had no significant in-

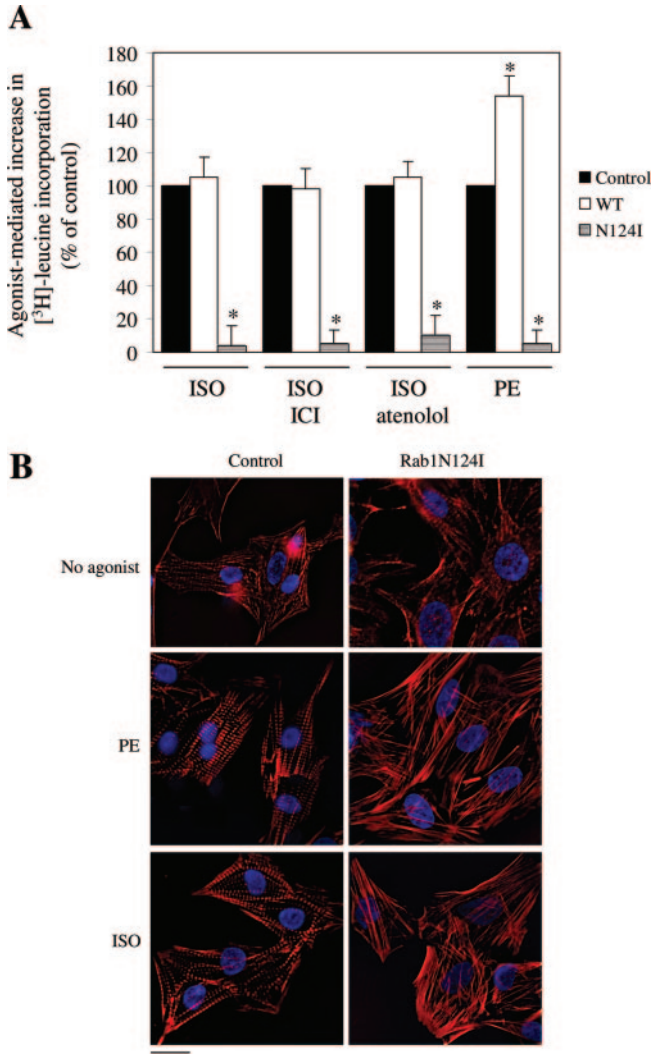

Fig. 6. Effects of Rab1 on β-AR- and α_1-AR-mediated hypertrophy in neonatal cardiomyocytes. A, the effect of adenoviral expression of Rab1 on total protein synthesis by activation of β-AR and α_1-AR. Cardiomyocytes were cultured in 12-well plates, infected with empty, Rab1WT or Rab1N124I adenoviruses (20 m.o.i.), and incubated with 1 μCi of [^{3}H]leucine for 24 h, and stimulated with ISO (10 μM), ISO plus ICI 118,551 (10 μM), ISO plus atenolol (10 μM) or PE (10 μM) for 24 h at 37°C. Total protein synthesis was measured as described under *Materials and Methods*. The data are shown as the -fold increase over the control and represent the means ± S.E. of three separate experiments each performed in triplicate. To reflect the effect of Rab1 on receptor agonist-mediated stimulation, enhancement of total protein synthesis by Rab1 itself was subtracted as described under *Materials and Methods*. *, $p < 0.05$ versus cardiomyocytes infected with control adenovirus. B, the effect of Rab1 on PE- and ISO-stimulated sarcomeric organization revealed by staining with phalloidin for F-actin. Similar results were obtained in three experiments. Scale bar, 10 μm.

fluence on the total cell surface number of β-AR. Consistent with total AR expression, the cell-surface numbers of α_{1A}-AR and α_{1B}-AR subtypes were largely increased, whereas Rab1 did not alter the cell-surface numbers of β_1-AR and β_2-AR subtypes. These data strongly indicate that increased Rab1 function facilitates the cell-surface targeting of α_1-AR subtypes but not β-AR subtypes. These data also suggest a novel way to selectively increase cell-surface expression of endogenous α_1-AR.

There are several possibilities regarding the selective regulation of β-AR and α_1-AR export to the cell surface by Rab1. First, the ER-to-Golgi transport of β-AR and α_1-AR may be mediated through distinct ER-derived vesicles, which are differentially regulated by Rab1. Consistent with this possibility, different proteins or isoforms have been reported to segregate into distinct vesicles (Muniz et al., 2001). Second, transport of β-AR and α_1-AR to the cell surface in cardiomyocytes may be mediated through distinct pathways, in which the level of endogenous Rab1 is a rate-limiting factor for the ER-to-Golgi transport of α_1-AR, but not β-AR. However, we cannot exclude the possibility that Rab1 also facilitated the ER-to-Golgi transport of β-AR, but did not significantly alter β-AR expression at the cell surface, because the Golgi-to-cell surface transport is a rate-limiting step for β-AR export. Third, structural differences in distinct ARs may contribute to their regulation by Rab1. We demonstrated previously that the transport of α_{2B}-AR from the ER to the cell surface in HEK293T cells is independent of Rab1 (Wu et al., 2003). Our present results indicate that the transport of endogenous α_1-AR and β-AR to the cell surface is dependent on Rab1 and that increased Rab1 function selectively facilitates the cell-surface targeting of α_1-AR subtypes. These data indicate that distinct ARs have different sensitivities to Rab1 manipulation. These ARs are structurally different, particularly at their C termini (C-terminal amino acid residues: α_{1A}-AR, 136; α_{1B}-AR, 174; β_1-AR, 96; β_2-AR, 88; α_{2B}-AR, 20). However, whether the C termini of ARs are indeed responsible for selective regulation by Rab1 is currently under investigation.

The present work demonstrated that Rab1-mediated ER-to Golgi transport is required for cell surface targeting of all AR subtypes examined (i.e., α_{1A}-, α_{1B}-, β_1-, and β_2-ARs). The cell-surface number of total β-AR and total α_1-AR as well as individual α_{1A}-AR, α_{1B}-AR, β_1-AR, and β_2-AR as measured by radioligand binding was significantly attenuated by adenoviral expression of the dominant-negative mutant Rab1N124I in cardiomyocytes. The subcellular localization of GFP-conjugated ARs reflects the effect of Rab1 on the newly synthesized receptors, in that GFP-receptors were delivered into the cell after Rab1 infection. It is noteworthy that Rab1 is one of the most extensively studied and best characterized Rab GTPases, which localize to the ER and the Golgi and regulate protein transport between these two organelles. Therefore, the influence of Rab1 on the cell surface receptor expression is presumably through modulating the ER-to-Golgi transport of the receptor.

Microscopic analysis of subcellular localization of GFP-conjugated receptors, which were delivered into cardiomyocytes after Rab1 infection, indicated expression of Rab1N124I induced an accumulation of α_{1B}-AR and β_2-AR in the ER. A similar behavior was observed when endogenous Rab1 was depleted using siRNA. Furthermore, BFA treatment, which impairs Golgi function and blocks the ER-to-Golgi protein transport (Klausner et al., 1992; Yoo et al., 2002), inhibited the transport of α_1-AR and β-AR to the cell surface and attenuated Rab1-induced enhancement of α_1-AR expression at the cell surface. These data suggest that β-AR and α_1-AR transport from the ER to the cell surface is mediated through the Rab1-dependend pathway and that Rab1 is involved in the transport from the ER to the Golgi.

Another important finding is that the functional response to β-AR and α_1-AR stimulation can be modulated by manipulating their transport along the early secretory pathway. Adenoviral expression of Rab1 markedly and selectively augmented ERK1/2 activation in response to stimulation with the α_1-AR agonist PE, but not to the β-AR agonist ISO in cardiomyocytes. Rab1N124I almost abolished the ERK1/2 activation by both α_1-AR and β-AR agonists. These data indicate that Rab1 can differentially regulate signaling of α1-AR and β-AR in cardiomyocytes, which was due to the influence of Rab1 on the transport of the receptors from the ER to the cell surface.

Present results also demonstrated that cardiomyocyte growth in response to the AR agonists can be controlled by manipulating the AR transport in the early secretary pathway. Consistent with the effect of Rab1 on the cell surface expression and signaling of β-AR and α_1-AR, Rab1 selectively promoted PE-mediated hypertrophy, as measured by changes in total protein synthesis in neonatal cardiomyocytes, and Rab1N124I attenuated both PE and ISO-stimulated cardiomyocyte hypertrophy. We previously demonstrated that, similar to α_1-AR, expression of Rab1 and Rab1N124I produced opposing effects on hypertrophic response to angiotensin II (Filipeanu et al., 2004). These data indicate that cardiomyocyte growth can be manipulated by controlling the transport of G protein-coupled receptors at the level of the ER and the Golgi compartment. It is noteworthy that the inhibitory effect of Rab1N124I on the agonist-promoted hypertrophic response (e.g., protein synthesis) and ERK1/2 activation was much more potent than on the cell-surface receptor number as determined by radioligand binding. It is unlikely that Rab1 GTPase, as a transport coordinator, is directly regulating signaling pathways. The differential influences of Rab1N124I on receptor cell-surface expression and agonist-activated signaling may suggest that the minimal amount of ARs at the cell surface is required for the agonist activation of the signaling pathways. When receptor expression at the cell surface is attenuated to a certain level by Rab1 expression, receptor-mediated signaling is almost totally blocked. If this turns out to be true, increased receptor expression would proportionally augment receptor-mediated signaling. Indeed, our data indicate that wild-type Rab1 expression increased receptor expression at the cell surface, agonist-mediated ERK1/2 activation and agonist-stimulated protein synthesis at the similar magnitudes. It is also possible that Rab1 regulates the export trafficking of other signaling molecules involved in the signal transduction systems of ARs.

We have shown that transgenic overexpression of Rab1 in the myocardium induces cardiac hypertrophy with progression to heart failure (Wu et al., 2001). However, the molecular mechanism responsible for Rab1-induced cardiomyocyte hypertrophy remains unknown. We have demonstrated that Rab1 promoted AT1R- and α_1-AR-mediated signaling and cardiomyocyte growth. These data suggest that one of the

possible molecular mechanisms underlying Rab1-induced hypertrophy in transgenic mouse hearts is that Rab1 overexpression activates signal transduction pathways of Gq-coupled AT1R and α_1-AR. This possibility is supported by the abilities of AT1R and α_1-AR activation to induce cardiomyocyte hypertrophic growth both in vivo animal hearts and in vitro cultured cardiomyocytes (Knowlton et al., 1993; Sadoshima and Izumo, 1993; Milano et al., 1994; Paradis et al., 2000).

In summary, we have shown for the first time a differential role of Rab1 in the transport and function of ARs, which are crucial for cardiac function under both normal and diseased conditions. Therefore, defining the functional role of export machinery (e.g., Rab1 GTPase) in cardiomyocytes by modifying the transport of selective G protein-coupled receptors in the early secretory pathway may provide a novel insight into understanding the regulation of these clinically important targets.

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Address correspondence to: Dr. Guangyu Wu, Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center, 1901 Perdido St., New Orleans, LA 70112. E-mail: gwu@lsuhsc.edu