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REGULATION OF THE TRAFFICKING AND FUNCTION OF G PROTEIN-COUPLED RECEPTORS BY RAB1 GTPASE IN CARDIOMYOCYTES

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Abstract

G protein-coupled receptors (GPCRs) play a crucial role in regulating cardiac growth and function under normal and diseased conditions. It has been well documented that the precise function of GPCRs is controlled by intracellular trafficking of the receptors. Compared with the extensive studies on the events of the endocytic pathway, molecular mechanism underlying the transport process of GPCRs from the endoplasmic reticulum (ER) through the Golgi to the cell surface and regulation of receptor signaling by these processes in cardiac myocytes remain poorly defined. This chapter describes the methods to characterize the function of Rab1 GTPase, which modulates protein transport from the ER to the Golgi apparatus, in the trafficking and signaling of angiotensin II receptor (AT1R), α_1 -adrenergic receptor (AR), and β -AR, and in hypertrophic growth in response to agonist stimulation in neonatal cardiac myocytes.

1. Introduction

G protein-coupled receptors (GPCRs) constitute a superfamily of cell surface receptors which, through coupling to distinct heterotrimeric G proteins, regulate downstream effectors such as adenylyl cyclases, phospholipases, protein kinases, and ion channels (Lefkowitz, 1996; Wess, 1998; Armbruster and Roth, 2005; Wu *et al.*, 1997, 1998, 2000a; Dong *et al.*, 2007). It has been well documented that GPCRs play a central role in regulating cardiac growth and function under both physiological and pathological conditions and a number of signaling molecules in the GPCR system are targets for treating cardiac disease (Post *et al.*, 1999; Lefkowitz *et al.*, 2000; Wu *et al.*, 2000b; Zhong and Neubig, 2001). The precise function of GPCRs and the magnitude of the agonist-elicited response are elaborately regulated by intracellular trafficking of the receptors (Duvernay *et al.*, 2005). However, compared with the extensive studies on the events involved in the endocytic pathway, the transport process of GPCRs from the endoplasmic reticulum (ER), where they are synthesized and properly assembled, through the Golgi to the cell surface and regulation of receptor signaling by export trafficking remain poorly defined. As an initial approach to understanding the molecular mechanisms underlying the export trafficking of GPCRs, we have investigated the function of Rab1 GTPase in regulating the cell-surface expression and signaling of $\alpha_2\text{B}$ -adrenergic receptor (AR), α_1 -AR, β_2 -AR and angiotensin II (Ang II) type 1A receptor (AT1R) (Wu *et al.*, 2003; Filipeanu *et al.*, 2004, 2006a), which couple to different heterotrimeric G-proteins and initiate distinct signal transduction pathways.

Rab1 is specifically localized in the ER and the Golgi apparatus and coordinates the export of newly synthesized proteins from the ER and subsequent anterograde transport from the ER to and through the Golgi stacks (Plutner *et al.*, 1991; Tisdale *et al.*, 1992; Nuoffer *et al.*, 1994; Allan *et al.*, 2000; Moyer *et al.*, 2001; Dugan *et al.*, 1995; Yoo *et al.*, 2002). There are two

isoforms, Rab1a and Rab1b, with greater than 90% identity in their amino acid sequences. We first used the dominant-negative Rab1 mutants and small interfering RNA as tools to explore Rab1 function in GPCR transport from the ER to the cell surface in HEK293T cells (Wu *et al.*, 2003). Our studies were then expanded to determine if the transport and signaling of endogenous GPCRs could be modified through manipulating Rab1 function in cardiomyocytes (including HL-1 atrial cardiomyocytes, primary cultures of neonatal cardiomyocytes, and the mouse heart genetically overexpressing Rab1a (Wu *et al.*, 2001; Filipeanu *et al.*, 2004, 2006a). In this chapter, we describe the methods to study the role of Rab1 GTPase in regulating the trafficking and signaling of GPCRs, particularly AT1R, α_1 -AR, and β -AR, and the hypertrophic response to their agonists in primary cultures of neonatal rat cardiomyocytes. At least two subtypes of β -ARs (β_1 - and β_2 -AR), two subtypes of α_1 -ARs (α_{1A} - and α_{1B} -AR), and two subtypes of Ang II receptors (the Ang II type 1 receptor [AT1R] and the Ang II type 2 receptor [AT2R]) have been identified in the mammalian heart. These receptors belong to the GPCR superfamily and play a crucial role in regulating cardiac growth and function under normal and diseased conditions (Post *et al.*, 1999; Filipeanu *et al.*, 2006b).

2. Materials

Human β_2 -AR, human α_{1B} -AR and rat AT1R were kindly provided by John D. Hildebrandt (Medical University of South Carolina), Kenneth P. Minneman, and Kenneth E. Bernstein (Emory University, Atlanta, GA), respectively. The receptors are tagged with green fluorescent protein (GFP) at their C-termini as described in Wu *et al.* (2003) and Hague *et al.* (2004). GFP as a tag is particularly useful for directly visualizing the subcellular localization and trafficking of proteins in the cell and has no substantial effect on the function and localization of many GPCRs (Kallal and Benovic, 2000). Rab1a is cloned from a mouse cardiac cDNA library (Wu *et al.*, 2001). The dominant-negative guanine nucleotide binding-deficient mutant Rab1aN124I is generated using QuikChange site-directed mutagenesis (Stratagene). Rab1a is tagged with the FLAG epitope at its N-terminus by polymerase chain reaction (PCR) using a primer GACTACAAGGACGACGATGACAAG coding a peptide DYKDDDDK (Wu *et al.*, 2003). [7-methoxy- 3 H]-prazosin (specific activity = 70 Ci/mmol), [3 H]-Ang II (50.5 Ci/mmol), [3 H]-CGP12177 (51 Ci/mmol), and [3 H]-leucine (173 Ci/mmol) are purchased from PerkinElmer Life Sciences (Boston, MA). Human Ang II is obtained from Calbiochem. Brefeldin A (BFA), PD123319, phenylephrine (PE), isoproterenol (ISO), atenolol, ICI 118,551, nifedipine, chloroethylclonidine (CEC), and anti-FLAG M2 monoclonal antibodies are from Sigma. Alexa Fluor 594-labeled phalloidin and 4,6-diamidino-2-phenylindole (DAPI) are obtained from Molecular Probes (Eugene, OR). Antibodies against Rab1 and phospho-ERK1/2 are purchased from Santa Cruz Biotechnology. Antibodies against ERK1/2 are from Cell Signaling Technology.

2.1. Rab1 regulation of GPCR expression at the plasma membrane in neonatal rat ventricular myocytes

Isolation and culture of neonatal rat ventricular myocytes, generation of adenoviruses expressing FLAG-Rab1a, and infection of myocytes by Rab1 adenoviruses are described in Filipeanu *et al.* (2007). Briefly, neonatal ventricular myocytes are isolated from the hearts of 1- to 2-day-old Sprague Dawley rats and cultured on 12-well plates at a density of 2×10^5 cells/well in DMEM medium supplemented with 10% fetal bovine serum (FBS) and antibiotics for 24 h. The myocytes are infected with control parent adenovirus or adenovirus expressing Rab1 or its dominant-negative mutant Rab1N124I at a multiplicity of infection (MOI) of 20 for 6 h, and then cultured in serum-free DMEM for 2 days. The number of receptors at the plasma membrane is measured by radioligand binding in intact live cardiomyocytes.

To measure the number of AT1R at the plasma membrane, the infected cardiomyocytes are incubated with PBS containing radiolabeled Ang II overnight. There are two types of radiolabeled Ang II, [^3H]-Ang II and [^{125}I]-Ang II. [^{125}I]-Ang II has a higher affinity for the Ang II receptors than [^3H]-Ang II (Bouscarel *et al.*, 1988; Matsubara *et al.*, 1994). Here we describe the method to use [^3H]-Ang II as a ligand to measure AT1R at the plasma membrane. There are several important points for measuring AT1R expression at plasma membrane by intact cell binding. First, the concentration of [^3H]-Ang II used for incubation should be optimized in initial experiments. This is true for all the ligand binding experiment. In our experiments, [^3H]-Ang II dose-dependent binding curve (2.5 to 20 nM) in cardiomyocytes infected with control parent adenovirus reveals that [^3H]-Ang II binding increases linearly at concentrations of 2.5 to 15 nM, and reaches maximal binding at the concentration of 15 nM. Therefore, cell-surface expression of AT1R in neonatal cardiomyocytes is measured at a saturating concentration of 20 nM. Second, as AT1R undergoes internalization in the presence of its agonist Ang II, the ligand binding assay is performed at 4°. Third, to exclude the contribution of AT2R to ligand binding, all solutions are supplemented with the specific AT2R antagonist PD123319 at a concentration of 1 μM . Finally, the nonspecific binding is determined in the presence of 10 μM of nonradioactive Ang II.

To measure the number of β -AR at the plasma membrane, the cells are incubated with PBS containing 10 nM of [^3H]-CGP12177, a hydrophobic β -AR ligand, for 90 min at room temperature. The nonspecific binding is determined in presence of alprenolol (20 μM). To measure expression of individual β -AR subtypes, myocytes are preincubated with the β_2 -AR-selective antagonists ICI 118,551 or β_1 -AR-selective antagonist atenolol (10 μM) for 30 min.

To measure the number of α_1 -AR at the plasma membrane, the infected myocytes are incubated with PBS containing 10 nM of [$^7\text{-Methoxy-}^3\text{H}$]-prazosin for 2 h at room temperature. To measure expression of individual α_1 -AR subtypes, myocytes are preincubated with the α_{1A} -AR-selective antagonist niguldipine or α_{1B} -AR-selective antagonist CEC at a concentration of 10 μM for 30 min. Nonspecific binding is determined in the presence of phentolamine (20 μM).

At the end of incubation, the myocytes are washed twice with 1 ml ice-cold PBS and the cell-surface-bound radioligand is extracted by mild acid treatment (2 $\times$ 5 min with 0.5 ml buffer containing 50 mM glycine, pH 3, and 125 mM NaCl) (Hunyady *et al.*, 2002). Alternatively, the cells are digested with 1 ml of 1 M NaOH. The radioactivity is counted by liquid scintillation spectrometry in 6 ml of Ecoscint A scintillation solution (National Diagnostics, Inc., Atlanta, GA).

It has been well demonstrated that treatment with BFA, a fungal metabolite that disrupts the structures of the Golgi (Klausner *et al.*, 1992), blocks protein transport from the ER to the Golgi. To determine if BFA treatment can attenuate Rab1-mediated receptor transport in cardiac myocytes, the Rab1-infected cardiomyocytes are incubated with BFA at a concentration of 5 $\mu\text{g/ml}$ for 8 h. Our data indicate BFA treatment significantly inhibits the Rab1-induced increase in AT1R expression at the cell surface.

In contrast to the ligand binding of membrane preparations which has been extensively used to quantify GPCR expression, intact cell ligand binding has at least two advantages. One is that intact cell ligand binding has the ability to accurately measure the number of receptors at the plasma membrane, compared with possible contamination with intracellular receptors of membrane preparations. The other is that intact cell ligand binding uses a relatively smaller quantity of cells, which is particularly important for primary cultures of cardiomyocytes. However, one issue associated with the radioligand binding of intact live cardiomyocytes is that radiolabeled ligands are often able to induce their receptor internalization (such as [^3H]-

Ang II for AT1R). One strategy to limit receptor internalization upon stimulation with the radiolabeled agonists is to carry out the experiment at low temperature as described for [³H]-Ang II binding. Low-temperature incubation (4°) limits AT1R internalization induced by the ligand Ang II during the binding as demonstrated by the fact that the amounts of radioligand obtained after NaOH digestion (to obtain total bound ligand) and acidic washing (to obtain cell-surface radioligand) are not significantly different. However, low-temperature incubation may reduce ligand binding to the receptors. For example, prazosin is a nonspecific α_1 -antagonist that undergoes internalization together with the receptor. However, our initial determinations performed at 4° failed to detect specific [³H]-prazosin binding (<500 cpm) in neonatal cardiomyocytes. In a typical experiment performed at room temperature, the specific binding of [³H]-prazosin obtained by digesting the cells with NaOH is about 2800 cpm, and nonspecific binding about 300 cpm, measured in the presence of phentolamine in neonatal cardiomyocytes. The specific binding of [³H]-prazosin recovered by successive acidic washings (representing the cell surface ligand) is about 2600 cpm. These data suggest that α_1 -AR internalization induced by the incubation with [³H]-prazosin (<10% of the total receptor) does not dramatically influence the cell-surface number of the receptor.

2.2. Rab1 regulation of the subcellular distribution of GPCRs in neonatal cardiomyocytes

Cardiomyocytes are grown on coverslips in six-well dishes and infected with control or Rab1 adenoviruses as above. After 6 h infection, the medium is removed and the myocytes are transiently transfected using LipofectAMINE 2000 reagent (Invitrogen) as described previously (Filipeanu *et al.*, 2004; Wu *et al.*, 2003). One microgram of GFP-tagged receptor is diluted into 125 μ l of serum-free Opti-MEM in a tube. In another tube, 5 μ l of LipofectAMINE is diluted into 125 μ l of serum-free Opti-MEM. Five min later both solutions are mixed and incubated for another 20 min. The transfection mixture is then added to culture dishes containing 0.8 ml of fresh Dulbecco's modified Eagle's medium. After transfection 36 to 48 h, the myocytes are fixed with a mixture of 4% paraformaldehyde and 4% sucrose in PBS for 15 min. The coverslips are mounted, and fluorescence is detected with a fluorescence microscope.

Based on the GFP signal, approximately 5 to 10% myocytes are transfected by this plasmid transfection protocol. As almost all the cardiomyocytes are infected by Rab1 adenovirus, the number of myocytes expressing GFP-tagged receptor achieved by transient transfection is sufficient to measure the effect of Rab1 on the subcellular localization of receptors in individual myocytes.

3. Effects

3.1. Effect of Rab1 on the signaling of GPCRs in cardiac myocytes

It is interesting to determine whether Rab1 is capable of regulating receptor function by modifying receptor trafficking. GPCRs activate multiple signaling pathways in cardiac myocytes, and any of these signaling systems can be chosen for this purpose. In our studies, we have determined the effect of Rab1 on the activation of the MAPK pathway, which is potently stimulated by AT1R, α_1 -AR, and β -AR in cardiac myocytes. Neonatal cardiomyocytes are cultured in six-well plates at a density of 5×10^5 cells/ well and infected with Rab1 constructs as described previously, and cultured in DMEM without serum for 48 h. The Rab1-infected myocytes are stimulated with 100 nM Ang II for 2 min (for AT1-mediated signaling), 10 μ M PE for 8 min (for α_1 -AR) or 10 μ M ISO for 8 min (for β -AR).

To block AT2R response to the Ang II stimulation, the myocytes are preincubated with 10 μ M PD123319 for 5 min. Similarly, to measure ERK1/2 activation by individual adrenergic receptors, the myocytes are preincubated with atenolol, ICI 118,551, nifedipine, and CEC at

a concentration of 10 μ M for 30 min to block the function of β_1 -AR, β_2 -AR, α_{1A} -AR, and α_{1B} -AR, respectively.

The reaction is terminated by the addition of 600 μ l of 1 $\times$ SDS gel loading buffer. After solubilizing the cells, 30 μ l of total cell lysate is separated by 10% SDS-PAGE and ERK1/2 activation measured by immunoblotting to determine 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.

3.2. Effect of Rab1 on the agonist-mediated protein synthesis in cardiomyocytes

The [3 H]-leucine incorporation is used to measure total protein synthesis in cardiomyocytes (Filipeanu *et al.*, 2007). Briefly, neonatal cardiomyocytes are cultured on 12-well plates at a density of 2×10^5 /well in DMEM supplemented with 10% FBS. After infection with the control and Rab1 adenoviruses, the myocytes are made quiescent by incubation for 48 h in DMEM without FBS. The cardiomyocytes are then incubated with [3 H]-leucine (1 μ Ci) for 12 h in the presence or absence of individual receptor agonists. The final concentrations of the receptor agonists are 100 nM Ang II, 10 μ M ISO and 10 mM PE. Similar to the measurement of ERK1/2 activation, the AT12R antagonist PD123319 is added together with Ang II at a final concentration of 10 μ M to block AT2R activation by Ang II stimulation. To block the function of individual adrenergic receptor β_1 -AR, β_2 -AR, α_{1A} -AR, and α_{1B} -AR, the myocytes are preincubated with atenolol, ICI 118,551, nifedipine, and CEC at a concentration of 10 μ M for 30 min, respectively. The reaction is terminated and protein synthesis determined by liquid scintillation spectrometry. Since Rab1 influences protein synthesis (Filipeanu *et al.*, 2004, 2007), the effect of Rab1 on receptor agonist-stimulated protein synthesis can be calculated using the following formula:

$$\frac{[\text{Ang II and Rab1}] - [\text{Rab1}]}{[\text{Ang II and control adenovirus}] - [\text{control adenovirus}]}$$

3.3. Effect of Rab1 on the agonist-mediated increase in size and sarcomeric organization in cardiomyocytes

The effect of adenoviral expression of Rab1 on the sizes of cardiac myocytes is determined by measuring cell surface area (Filipeanu *et al.*, 2004, 2007). The isolated myocytes are cultured on coverslips in six-well dishes at a density of 1×10^4 cells/well, and made quiescent in DMEM without FBS for 48 h and infected with control and Rab1 adenoviruses. The myocytes are stimulated with the receptor agonists: 100 nM Ang II, 10 μ M ISO, and 10 μ M PE for 24 h. After the myocytes are fixed and stained with Alexa Fluor-594 conjugated phalloidin, the cell surface is measured (Filipeanu *et al.*, 2007). The effect of Rab1 on the receptor agonist-mediated increase in cell size is calculated using the same formula as for the protein synthesis described previously.

4. Experimental Results and Concluding Remarks

Using the methods described in this chapter, we have demonstrated that adenoviral expression of the dominant negative mutants Rab1N124I attenuates the cell-surface expression of all GPCRs examined including AT1R, α_{1A} -AR, α_{1B} -AR, β_1 -AR, and β_2 -AR in neonatal cardiac myocytes. Interestingly, augmentation of Rab1 function by adenoviral expression of Rab1 facilitates the cell-surface expression of AT1R, α_{1A} -AR, and α_{1B} -AR, without altering β_1 -AR and β_2 -AR expression (Fig. 16.1A) (Filipeanu *et al.*, 2004, 2006a). These data indicate that the transport to the cell surface of endogenous GPCRs is differentially regulated by Rab1 in cardiac myocytes. Subcellular localization analysis revealed that the receptors were unable to transport to the cell surface and were trapped inside the cells infected with Rab1N124I adenoviruses (see Fig. 16.1B) (Filipeanu *et al.*, 2004, 2006a). Consistent with Rab1 effect on the receptor

expression at the cell surface, ERK1/2 activation in response to stimulation with receptor agonists is inhibited in neonatal cardiomyocytes infected with adenovirus expressing Rab1N124I, and adenoviral expression of Rab1 selectively promotes ERK1/2 activation by Ang II and PE, but not ISO (Filipeanu *et al.*, 2004, 2006a). Similarly, Rab1N124I attenuates cardiomyocyte hypertrophic response to all the agonists, whereas Rab1 selectively promotes cardiomyocyte hypertrophic response to Ang II (Fig. 16.2) and PE, but not ISO (Filipeanu *et al.*, 2004, 2006a). These data provide strong evidence implicating that GPCR function can be modulated through manipulating their traffic from the ER to the Golgi, which in turn alters the responsiveness of cardiomyocytes to the receptor stimulation.

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Figure 16.1.

Effect of adenovirus-mediated expression of Rab1 and its dominant negative mutant Rab1N124I on the cell surface expression (A) and subcellular localization (B) of GPCRs in neonatal cardiomyocytes. (A) Cardiomyocytes were cultured and infected with control, Rab1, or Rab1N124I adenovirus for 2 days at a multiplicity of infection of 20. The cell-surface expression of AT1R, α_1 -AR, and β -AR was determined by the radioligand [3 H]-Ang II, [3 H]-prazosin, and [3 H]-CGP12177, respectively. The data shown are the percentage of the mean value obtained from the cardiomyocytes infected with control adenovirus (the dotted line).

* $p < 0.05$ versus control. (B) Cardiomyocytes were grown on coverslips, infected with control or Rab1N124I adenoviruses, and transiently transfected with GFP-tagged β_2 -AR. Two days after infection, the myocytes were fixed and stained with 4,6-diamidino-2-phenylindole (nuclear). Blue, nuclear stained by 4,6-diamidino-2-phenylindole; green, GFP- β_2 -AR. (From Filipceanu, C. M., Zhou, F., Claycomb, W. C., and Wu, G. (2004). Regulation of the cell surface

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Figure 16.2.

Effect of adenovirus-mediated expression of Rab1 and its dominant negative mutant Rab1N124I on Ang II-stimulated hypertrophic response in neonatal cardiomyocyte. To reflect the effect of Rab1 on Ang II-mediated stimulation, the contribution of Rab1 itself to protein synthesis and cell surface area was subtracted. Cardiomyocytes were cultured and infected with control, Rab1, and Rab1N124I adenoviruses for 2 days, and then stimulated with Ang II (100 nM) for 24 h. (A) Effect of Rab1 on Ang II-stimulated total protein synthesis measured by [3H]-leucine incorporation. (B) Effect of Rab1 on Ang II-mediated increase in cell surface area. (C) Effect of Rab1N124I on Ang II-stimulated sarcomeric organization revealed by staining with phalloidin for F-actin. * $p < 0.05$ versus control. (Adapted from From Filipeanu, C. M., Zhou, F., Claycomb, W. C., and Wu, G. (2004). Regulation of the cell surface expression and function of angiotensin II type 1 receptor by Rab1-mediated endoplasmic reticulum-to-Golgi transport in cardiac myocytes. *J. Biol. Chem.* **279**, 41077–41084.)