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The GTP-binding Protein RhoA Mediates Na,K-ATPase Exocytosis in Alveolar Epithelial Cells

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The purpose of this study was to define the role of the Rho family of small GTPases in the β-adrenergic regulation of the Na,K-ATPase in alveolar epithelial cells (AEC). The β-adrenergic receptor agonist isoproterenol (ISO) increased the Na,K-ATPase protein abundance at the plasma membrane and activated RhoA in a time-dependent manner. AEC pretreated with mevastatin, a specific inhibitor of prenylation, or transfected with the dominant negative RhoAN19, prevented ISO-mediated Na,K-ATPase exocytosis to the plasma membrane. The ISO-mediated activation of RhoA in AEC occurred via β_2-adrenergic receptors and involved G_s-PKA as demonstrated by incubation with the protein kinase A (PKA)-specific inhibitors H89 and PKI (peptide specific inhibitor), and G_i, as incubation with pertussis toxin or cells transfected with a minigene vector for G_i inhibited the ISO-mediated RhoA activation. However, cells transfected with minigene vectors for G_{12} and G_{13} did not prevent RhoA activation by ISO. Finally, the ISO-mediated Na,K-ATPase exocytosis was regulated by the Rho-associated kinase (ROCK), as preincubation with the specific inhibitor Y-27632 or transfection with dominant negative ROCK, prevented the increase in Na,K-ATPase at the plasma membrane. Accordingly, ISO regulates Na,K-ATPase exocytosis in AEC via the activation of β_2-adrenergic receptor, G_s, PKA, G_i, RhoA, and ROCK.

INTRODUCTION

β-adrenergic receptors are members of the large family of seven membrane-spanning, GTP-binding protein-coupled receptors (GPCRs). In response to agonists, specific domains of the GPCRs interact with heterotrimeric GTP-binding proteins leading to the exchange of GTP for GDP, resulting in the dissociation of the heterotrimer into active Gα- and Gβγ-subunits (Rockman *et al.,* 2002). It is commonly assumed that β-adrenergic receptor agonists promote the activation of the stimulatory G protein, G_s, which in turn increases the activity of adenylyl cyclase, increasing the cellular levels of cAMP and the phosphorylation, via cAMP-dependent protein kinase A (PKA), of downstream proteins. In addition to G_s activation, β2-adrenergic receptors have been shown to be coupled to the pertussis toxin (PTX)-sensitive heterotrimeric G protein, G_i (Daaka *et al.*, 1997; Post *et al.*, 1999; Gosmanov *et al.,* 2002).

β-adrenergic receptor agonists increase lung edema clearance by upregulating the Na,K-ATPase in the alveolar epithelium (Berthiaume *et al.,* 1987; Suzuki *et al.,* 1995; Saldias *et al.,* 1998; Saldias *et al.,* 1999, 2000; Sznajder *et al.,* 2002; Ridge *et al.,* 2003). The Na,K-ATPase located at the basolateral plasma membrane (BLM) of alveolar epithelial cells (AEC) generates, via the vectorial transport of Na^+, the gradient necessary for the movement of water from the alveolar space into the pulmonary circulation (Rutschman *et al.,* 1993; Saumon and Basset, 1993; Sznajder *et al.,* 1995; Ridge *et al.,* 2003). Activation of β-adrenergic receptors by isoproterenol (ISO) in AEC resulted in increased Na,K-ATPase activity due to the translocation of Na,K-ATPase molecules from intracellular compartments to the BLM via a process that is dependent on the actin cytoskeleton (Bertorello *et al.,* 1999; Ridge *et al.,* 2003).

Studies in secretory cells have shown that remodeling of the actomyosin cortex is a prerequisite for regulated exocytosis (Lang *et al.,* 2000; Oheim and Stühmer, 2000). The Rho family of GTPases is thought to have a central role in vesicular trafficking pathways by controlling the organization of the actin cytoskeleton to spatially direct the transport of vesicles (Guo *et al.,* 2001). Rho GTPases act as molecular switches cycling between GDP- and GTP-bound states. When bound to GDP they are inactive; upstream events lead to the exchange of GDP for GTP and the protein switches into an active conformation (Van-Aelst and D'Souza-Scho-

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rey, 1997; Kjøller and Hall, 1999). Rho in its inactive state is cytosolic and complexed with GDIs (guanine nucleotide dissociation inhibitors; Fukumoto *et al.,* 1990). Receptor-mediated activation leads to recruitment of Rho to the plasma membrane, where it gets anchored through a geranylgeranyl lipid residue that is attached to the C terminus. Once at the plasma membrane, through the action of GEFs (guanine nucleotide exchange factors), the GTP loading occurs (Cherfils and Chardin, 1999).

The present study was conducted to determine whether RhoA participated in the β-adrenergic receptor-mediated exocytosis of the Na,K-ATPase in AEC. The results demonstrate that ISO, via β_2-adrenergic receptors, G_s-PKA and G_i, activates RhoA and that RhoA via Rho-associated kinase has an important regulatory role in the β-adrenergic-mediated Na,K-ATPase exocytosis in AEC.

MATERIALS AND METHODS

Reagents

$^{86}Rb^+$ was purchased from Amersham Pharmacia (Piscataway, NJ). PKA inhibitor peptide myristoylated, PTX, and Rho-associated kinase inhibitor, Y-27632, were from Calbiochem (La Jolla, CA). All other chemicals were purchased from Sigma (St. Louis, MO). The Na,K-ATPase α_1 mAb (clone 464.6) and antiphospho-MYPT1 (Thr696) polyclonal antibody were from Upstate Biotechnolgy (Lake Placid, NY). RhoA mAb (clone 26C4) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Myosin phosphatase target subunit (MYPT) polyclonal antibody was from BabCO (Berkeley Antibody Company, Richmond, CA). Secondary goat anti-mouse HRP and goat anti-rabbit HRP were from Bio-Rad (Hercules, CA).

Cell Culture

A549 cells (ATCC CCL 185, a human adenocarcinoma cell line) were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded in 6- or 10-cm plates, grown to confluence, and serum-starved 18–24 h before treatments. A549 cells has been proven to be a good model for the study of Na^+-transport of AEC (Lazrak *et al.,* 2000; Lecuona *et al.,* 2000).

Permanent Transfection

A549 cells were plated in 6-cm plates at 2–3 × 10^5 cells/plate and transfected with 2 μg of plasmid DNA (dominant negative [dn] RhoA [RhoAN19], a gift of Dr. S. Gutkind, NIH; minigenes for G_i, G_{12}, and G_{13}, a gift from Dr. H. Hamm, Vanderbilt University) by using Superfect Reagent (Qiagen, Hilden, Germany), as indicated by the manufacturer. Transfectants were selected in the presence of 400 μg/ml geneticin (G418; Mediatech, Herndon, VA). Individual colonies were isolated using cloning cylinders (PGC Scientifics, Frederick, MD), and the resulting cell lines were propagated in complete DMEM supplemented with G418.

Transient Transfection

A549 cells were plated in 6-cm plates at 5 × 10^5 cells/plate and transfected with 5 μg of plasmid DNA (dominant negative [ROCK KD-IA]] and activated [ROCK Δ 3] forms of ROCK, a gift of Dr. S. Narumiya, Kyoto University) by using Lipofectin Reagent (Life Technologies, Gaithersburg, MD), as indicated by the manufacturer. Cells were used 48 h posttransfection.

1% Triton X-100–soluble Fraction

Cells were treated with different agonists/antagonists at 37°C, placed on ice, and washed twice with ice-cold phosphate-buffered saline (PBS). Cells were scraped in PBS, centrifuged, resuspended in homogenization buffer (1 mM EDTA, 1 mM EGTA, 10 mM Tris-HCl, pH 7.5, 1 μg/ml leupeptin, 100 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone [TPCK] and 1 mM phenylmethylsulfonyl fluoride [PMSF]), and homogenized. Homogenates were centrifuged at 100,000 × *g*, 1 h, 4°C (TL ultracentrifuge, Beckman, Fullerton, CA; Rotor TLA 100.2), and the pellet containing the crude membrane fraction was resuspended in homogenization buffer + 1% Triton X-100 and centrifuged at 100,000 × *g*, 30 min, 4°C. The supernatant was considered as the 1% Triton X-100–soluble fraction.

Biotinylation of Cell Surface Proteins

Cells were treated with different agonists/antagonists at 37°C, placed on ice, and washed twice with ice-cold PBS, and surface proteins were labeled for 1 h using 0.5 mg/ml EZ-link NHS-SS-biotin (Pierce Chemical Co., Rockford, IL). After labeling, the cells were rinsed three times with PBS containing 50 mM glycine to quench unreacted biotin and then lysed in modified RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, and 1% sodium deoxycholate, 1 μg/ml leupeptin, 100 μg/ml TPCK, and 1 mM PMSF). Proteins, 150–300 μg, were incubated overnight at 4°C with end-over-end shaking in the presence of streptavidin beads (Pierce Chemical Co., Rockford, IL). Beads were thoroughly washed (Carranza *et al.,* 1998), resuspended in 30 μl of Laemmli's sample buffer solution (Laemmli, 1970) and analyzed by Western blot.

Western Blot Analysis

Protein was quantified by Bradford assay (Bradford, 1976; Bio-Rad, Hercules, CA) and resolved in 10–15% polyacrylamide gels. Thereafter, proteins were transferred onto nitrocellulose membranes (Optitran, Schleicher & Schuell, Keene, NH) using a semidry transfer apparatus (Bio-Rad). Incubation with specific antibodies was performed overnight at 4°C. Blots were developed with a chemiluminescence detection kit (PerkinElmer Life Sciences, Boston, MA) used as recommended by the manufacturer. The bands were quantified by densitometric scan (Eagle Eye II, Stratagene, La Jolla, CA).

RhoA Pull-down Assay

The assay was performed using the Rho activation assay kit (Upstate Biotechnology, Lake Placid, NY) as recommended by the manufacturer. The assay is based in the method recently described by Ren *et al.* (1999), where cellular GTP-Rho is detected by Western blot after affinity precipitation with a fusion protein containing GST and the Rho binding domain of Rhotekin (GST-RBD).

$^{86}Rb^+$ Uptake

The assay was run at 37°C in a reciprocating bath at 100 rpm. Cells were preincubated in serum-free DMEM, containing HEPES with or without 100 μM ouabain and 10 μM ISO for 10 min, and then the medium was removed and fresh medium containing 1 μCi/ml $^{86}Rb^+$, with or without 100 μM ouabain and 1 μM ISO, was added. After a 5-min incubation, uptake was terminated by aspirating the assay medium and washing the plates in 4°C $MgCl_2$. Cells were air-dried and extracted with 0.1% NaOH, and $^{86}Rb^+$ influx was quantified by liquid scintillation counting. Initial influx, expressed as micromoles of K^+/μg of protein/min, is calculated as follows:

$$influx = (cpm/\mu gprotein/5min)/SA_{ex}$$

where SA_{ex} is the specific activity of the extracellular phase (cpm/μmol K^+). Ouabain inhibitable fraction of the Na,K-ATPase was measured as $^{86}Rb^+$ uptake in the presence of 100 μM ouabain.

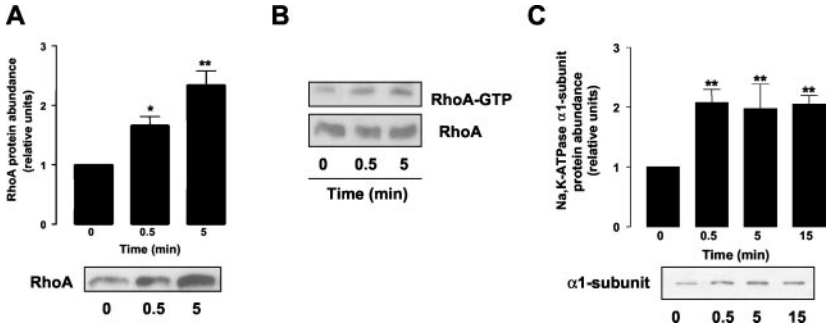

Figure 1. ISO activates RhoA and Na,K-ATPase in A549 cells. (A) A549 cells were incubated with 10 μM ISO for 0, 0.5, and 5 min; 1% Triton X-100–soluble fractions were obtained and RhoA translocation was evaluated by Western blot. Top: bars represent mean ± SEM (n = 6). *$p < 0.05$; **$p < 0.01$. Bottom: a representative Western blot. (B) A549 cells were incubated with 10 μM ISO and cell lysates were subjected to a pull-down assay with GST-RBD. A representative Western blot of RhoA bound to GTP (top) and total RhoA (bottom) is shown (n = 3). (C) A549 cells were incubated with 10 μM ISO, and Na,K-ATPase exocytosis was studied by biotin labeling of surface proteins followed by streptavidin pull-down and Western blot analysis using a specific antibody. Top: bars represent mean ± SEM (n = 4). **$p < 0.01$. Bottom: a representative Western blot.

Immunofluorescence

Cells, 2.5×10^5, were grown over glass coverslips, allowed to attach, and serum-starved for 18–24 h before adding 100 μM lysophosphatidic acid (LPA) for 20 min. Then cells were fixed with 2% formaldehyde in PBS for 20 min at room temperature. After permeabilization with 1% Triton X-100 and blocking with 0.2% BSA and normal goat serum, A549 cells were stained with rhodamine-phalloidin to study stress fiber formation. Cells were visualized under fluorescence microscope (Nikon Eclipse E800, Fryer Company, Huntley, IL).

Myosin Phosphatase Target Subunit Immunoprecipitation

Cells were treated with different agonists/antagonists at 37°C, placed on ice and washed twice with ice-cold PBS. Cells were scraped in immunoprecipitation buffer (20 mM Tris-HCl, 2 mM EGTA, 2 mM EDTA, 30 mM $Na_4P_2O_7$, 30 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, pH 7.4), frozen in liquid nitrogen, thawed, sonicated, frozen again, and centrifuged for 2 min at 14,000 × *g*. After protein determination, 0.2% SDS and 1% Triton X-100 was added to each sample. Equal amounts of protein (600–900 μg) were then incubated with anti-MYPT antibody for 2 h at 4°C. Protein A/G PLUS Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added, and the samples were incubated overnight at 4°C. The samples were then washed three times with IP buffer supplemented with 0.2% SDS and 1% Triton X-100 and once with 250 mM Tris, pH 7.4. A Western blot was performed using phospho-MYPT (Thr696). Membranes were then stripped with stripping buffer (2% SDS, 100 mM 2-mercaptoethanol, 62.5 mM Tris, pH 6.8) and reprobed using an MYPT antibody.

Statistical Analysis

Data are presented as means ± SEM. Comparisons were made using a one-way analysis of variance followed by a multiple comparison test (Dunnett) when the *F* statistic indicated significance. Results were considered significant when $p < 0.05$.

RESULTS

ISO-activated RhoA and Na,K-ATPase in A549 Cells

To determine whether RhoA was activated by β-adrenergic agonists, we performed a time course, incubating A549 cells with 10 μM ISO and assessed RhoA translocation to the 1% Triton X-100–soluble fraction by Western blot, a hallmark of RhoA activation (Fleming *et al.*, 1996). As depicted in Figure 1A, ISO stimulated RhoA translocation to the Triton X-100–soluble fraction within 30 s of treatment. ISO-mediated activation of RhoA was also determined using a pull-down assay (Ren *et al.*, 1999). As shown in Figure 1B, top panel, A549 cells treated with ISO had a time-dependent increased recovery of RhoA bound to GTP (active) compared with control. The total amount of RhoA in the cell lysates was unchanged in control vs. ISO-treated A549 cells, indicating the specificity of the increase in the pull-down assay (Figure 1B, bottom panel). The time course of RhoA activation paralleled the translocation of Na,K-ATPase from intracellular compartments to the plasma membrane as shown in Figure 1C.

Mevastatin and dn-RhoA Prevented ISO-mediated Na,K-ATPase Exocytosis

3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors prevent RhoA isoprenylation, thus impairing RhoA membrane localization and activation (Laufs *et al.*, 1999; Takeuchi *et al.*, 2000). Therefore, to study the role of RhoA in Na,K-ATPase exocytosis, we pretreated A549 cells with the HMG-CoA reductase inhibitor mevastatin (10 μM, 16 h). Mevastatin treatment prevented both the ISO-mediated RhoA translocation to the 1% Triton X-100–soluble fraction (Figure 2A) and the ISO-mediated Na,K-ATPase exocytosis (Figure 2B).

To further demonstrate a role for RhoA in ISO-mediated Na,K-ATPase regulation, we used A549 cells permanently transfected with a dn-RhoA. The dn-RhoA clones were characterized by assessing stress fiber formation induced by incubation with lysophosphatidic acid (LPA, 100 μM, 20 min), a known RhoA-mediated phenomenon (Ridley and Hall, 1992). As depicted in Figure 3A, formation of stress fibers was observed in control cells after LPA treatment, but not in the A549 cells expressing the dn-RhoA (RhoA N19).

A549 cells permanently expressing dn-RhoA had the same basal Na,K-ATPase activity (assessed by $^{86}Rb^+$ uptake) as control A549 cells or cells transfected with the empty vector, but failed to increase Na,K-ATPase activity or exocytosis of the Na,K-ATPase to the plasma membrane upon ISO stimulation (Figure 3, B and C).

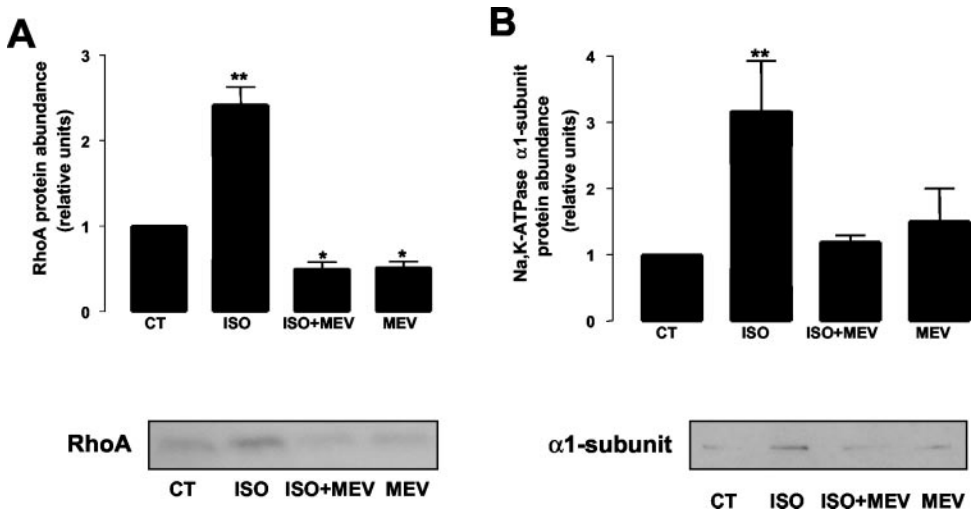

Figure 2. Mevastatin prevents ISO-mediated Na,K-ATPase exocytosis. A549 cells were incubated with 10 μM mevastatin for 16 h before ISO stimulation. (A) RhoA translocation to the 1% Triton X-100–soluble fraction was studied by Western blot using a specific antibody. Top: bars represent mean ± SEM (n = 4). *p <0.05; **p <0.01. Bottom: a representative Western blot. (B) Na,K-ATPase exocytosis was studied by biotin labeling of surface proteins followed by streptavidin pull-down and Western blot analysis using a specific antibody. Top: bars represent mean ± SEM (n = 4). **p < 0.01. Bottom: a representative Western blot.

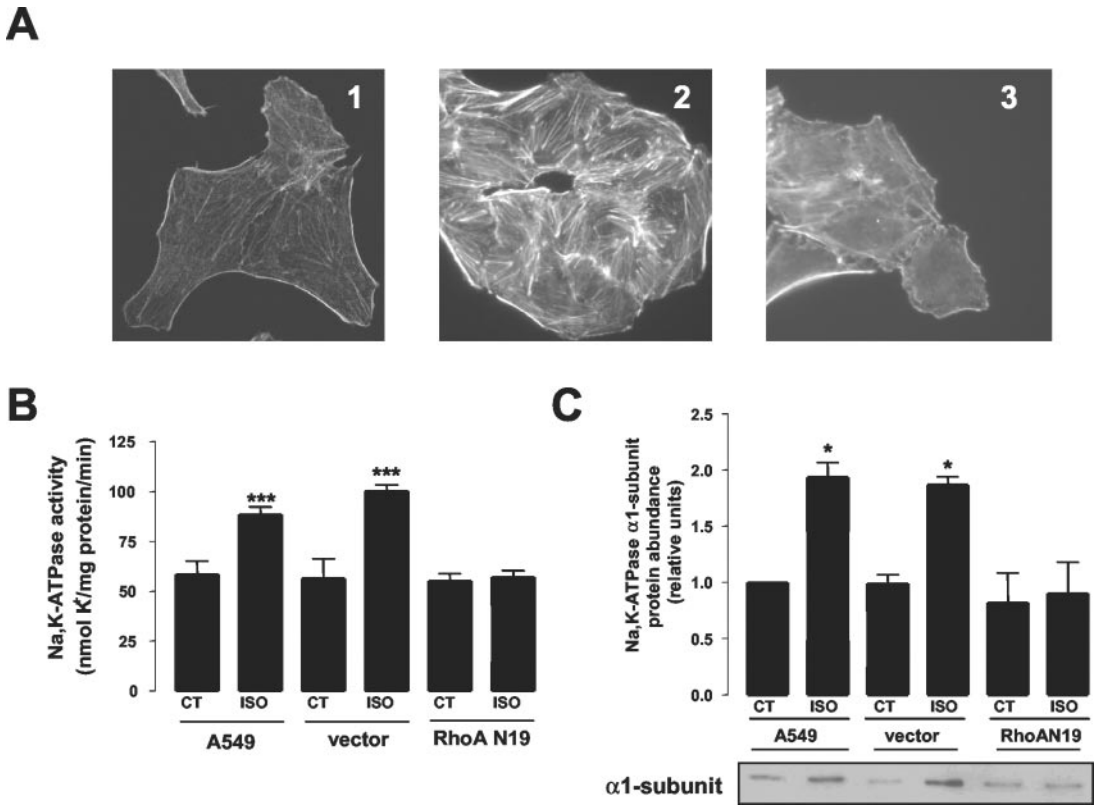

Figure 3. Dominant negative RhoA prevents isoproterenol-mediated Na,K-ATPase exocytosis. Permanently transfected A549 cells expressing either vector or dominant negative RhoA (RhoAN19) were grown in the presence of selection. (A) Actin stress fiber formation was assessed in cells fixed and stained with rhodamin-palloidin and evaluated by using fluorescence microscopy. Representative photomicrographs of control cells (1), and control cells (2), and cells transfected with RhoAN19 (3) treated with 100 μM lysophosphatidic acid (LPA) for 20 min are shown. (B) Na,K-ATPase activity, measured as $^{86}Rb^{+}$ uptake, in control cells and cells transfected with the vector or RhoAN19, after incubation with 10 μM ISO for 15 min. Top: bars represent mean ± SEM (n = 4). ***p < 0.001. (C) Na,K-ATPase exocytosis was studied by biotin labeling of surface proteins followed by streptavidin pull-down and Western blot analysis using a specific antibody. Top: bars represent mean ± SEM (n = 4). **p < 0.01. Bottom: a representative Western blot.

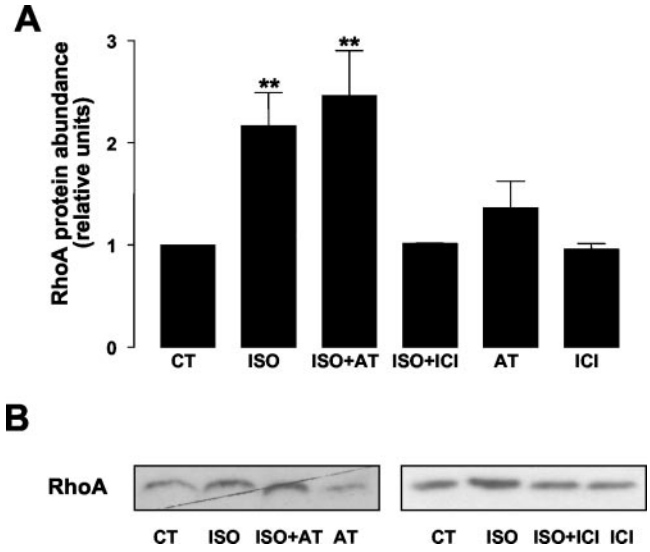

Figure 4. ISO-mediated RhoA activation occurs via β_2-adrenergic receptors. A549 cells were incubated with 100 μM atenolol (β_1-adrenergic receptor antagonist) or 10 μM ICI 118,551 (β_2-adrenergic receptor antagonist) before adding 10 μM ISO for 15 min. 1% Triton X-100–soluble fraction was isolated and Western blot against RhoA was performed. (A) Bars represent mean ± SEM (n = 4). **$p < 0.01$. (B) A representative Western blot.

ISO-activated RhoA via β_2-Adrenergic Receptors, cAMP-PKA, and G_i

To determine which β-adrenergic receptor was mediating RhoA stimulation, we pretreated A549 cells with specific β_1-adrenergic receptor (atenolol, 100 μM, 30 min) and β_2-adrenergic receptor (ICI 118,551, 10 μM, 30 min) antagonists. As shown in Figure 4, ISO-mediated RhoA translocation to the 1% Triton X-100–soluble fraction was prevented in cells pretreated with the β_2-adrenergic receptor antagonist ICI 118,551, but not in cells pretreated with the β_1-adrenergic receptor antagonist atenolol.

The β_2-adrenergic receptor is known to couple to G_s, stimulate adenylyl cyclase, and activate the cyclic-AMP-dependent protein kinase (PKA) (Daaka *et al.*, 1997; Post *et al.*, 1999). PKA has been described to play a role in the β-adrenergic regulation of Na,K-ATPase in AEC (Bertorello *et al.*, 1999). Thus, to determine whether the ISO-mediated activation of RhoA occurred via PKA, we pretreated the cells with 1 μM H89 (specific PKA inhibitor) or with a myristoylated peptide that specifically inhibits PKA (PKI, 10 μM) for 30 min before treatment with 10 μM ISO for 5 min. As shown in Figure 5A, incubation with H89 (top panel) or with PKI (bottom panel) blocked the ISO-mediated RhoA translocation to the 1% Triton X-100–soluble fraction. This result was further confirmed by the use of the adenylyl cyclase activator forskolin (50 μM, 5 min), which translocated RhoA to the 1% Triton X-100–soluble fraction (Figure 5B).

β_2-adrenergic receptors are coupled to the PTX-sensitive heterotrimeric G protein, G_i (Daaka *et al.*, 1997; Post *et al.*,

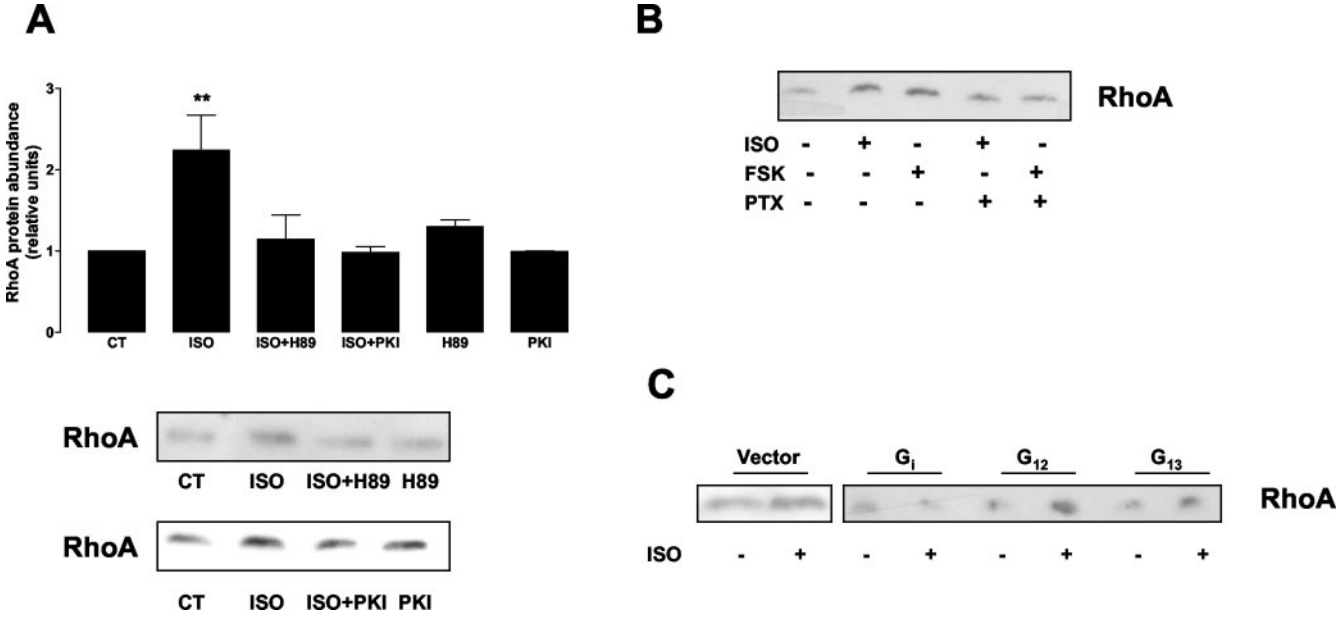

Figure 5. RhoA is activated via G_s-PKA and G_i heterotrimeric G proteins. (A) A549 cells were incubated with 1 μM H89 or 10 μM PKI for 30 min before adding 10 μM ISO. 1% Triton X-100–soluble fractions were isolated, and Western blot against RhoA was performed. Top: bars represent mean ± SEM (n = 3). **$p < 0.01$. Bottom: a representative Western blot. (B) A549 cells were incubated with 100 ng/ml PTX for 16 h before adding 10 μM ISO or 50 μM forskolin for 5 min. 1% Triton X-100–soluble fractions were isolated, and Western blot against RhoA was performed. A representative Western blot is shown (n = 3). (C) A549 cells were permanently transfected with empty vector or minigene vectors expressing Gα COOH-terminal peptides for G_i, G_{12}, and G_{13}. Cells were incubated with 10 μM ISO for 5 min, 1% Triton X-100–soluble fractions were isolated, and Western blot against RhoA was performed. A representative Western blot is shown (n = 3).

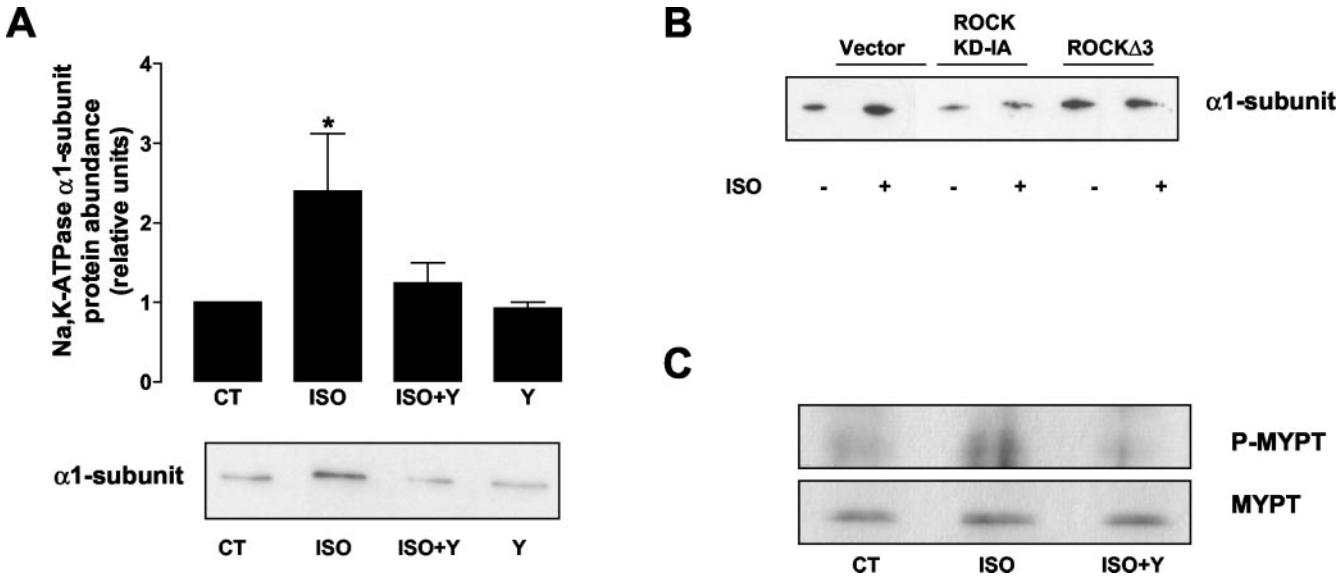

Figure 6. ROCK/MYPT are involved in the β-adrenergic receptor induced Na,K-ATPase exocytosis.(A) A549 cells were incubated with 10 μM Y-27632 before incubating with 10 μM ISO for 15 min. Na,K-ATPase exocytosis was studied by biotin labeling of surface proteins followed by streptavidin pull-down and Western blot analysis using a specific antibody. Top: bars represent mean ± SEM (n = 3). *$p < 0.05$. Bottom: a representative Western blot. (B) A549 cells were transiently transfected with vector, dominant negative ROCK (ROCK KD-IA), or activated ROCK (ROCKΔ 3) and Na,K-ATPase exocytosis was studied by biotin labeling of surface proteins. A representative Western blot is shown (n = 3). (C) A549 cells were incubated with 10 μM ISO in the presence or absence of Y-27632. The cell lysates were immunoprecipitated with polyclonal MYPT antibody. The resulting immunoprecipitates were then immunoblotted with either phospho-MYPT (Thr696) antibody or pan-MYPT antibody. A representative Western blot is shown (n = 3).

1999; Gosmanov *et al.,* 2002). To examine whether G_i proteins play a role in ISO-mediated RhoA activation, we pretreated A549 cells with 100 ng/ml PTX for 16 h before stimulating with ISO or forskolin for 5 min. As shown in Figure 5B, PTX prevented RhoA translocation to the 1% Triton X-100–soluble fraction of both ISO- and forskolin-stimulated cells.

Several RhoA-regulated processes are mediated by heterotrimeric G proteins of the $G_{12/13}$ family (Buhl *et al.,* 1995; Fromm *et al.,* 1997; Hart *et al.,* 1998; Mao *et al.,* 1998). Thus, to determine whether $G_{12/13}$ were involved in ISO-mediated RhoA activation, we generated A549 cells permanently transfected with minigene vectors expressing Gα COOH-terminal peptides for G_i (to confirm the PTX data), G_{12} and G_{13} (Gilchrist *et al.,* 2002a, 2002b). As shown in Figure 5C, ISO-mediated activation of RhoA was not affected in A549 cells expressing the minigene for G_{12} and G_{13}. However, in cells expressing the minigene for G_i, RhoA activation by ISO was prevented. Taken together these results suggest a role for β_2-adrenergic receptor, G_s, PKA and G_i in the upstream pathway leading to RhoA stimulation by ISO.

Downstream Effectors of RhoA

We also studied whether two well-known downstream effectors of RhoA, Rho-associated kinase (ROCK) and phospholipase D (PLD), had a role in the ISO-mediated Na,K-ATPase exocytosis. To study ROCK involvement, A549 cells were treated with the ROCK-specific inhibitor Y-27632 (10 μM, 30 min) before incubation with 10 μM ISO for 15 min. As shown in Figure 6A, incubation with Y-27632 prevented ISO-induced Na,K-ATPase exocytosis. To further confirm the involvement of ROCK in the ISO-induced Na,K-ATPase exocytosis, we transiently transfected A549 cells with plasmids coding for dominant negative (ROCK KD-IA) and activated (ROCK Δ 3) forms of ROCK (Ishizaki *et al.,* 1997). As shown in Figure 6B, cells expressing ROCK KD-IA were unable to induce the translocation of the Na,K-ATPase to the plasma membrane after ISO stimulation, whereas cells expressing the activated form of ROCK had an increased amount of Na,K-ATPase at the plasma membrane in control conditions that could not be further increased after ISO treatment. These data strongly suggest a role for ROCK as a downstream effector of RhoA in the ISO-induced Na,K-ATPase exocytosis in AEC.

Activated ROCK regulates the phosphorylation of myosin light chain in part by the inactivation of myosin phosphatase through the phosphorylation of its regulatory subunit MYPT (Kimura *et al.,* 1996). To determine whether MYPT was a direct target for ROCK in the β-adrenergic regulation of AEC, A549 cells were incubated with 10 μM ISO in the presence or absence of the ROCK inhibitor Y-27632, and phosphorylation of MYPT at Thr696 (Feng *et al.,* 1999) was examined by immunoprecipitation of MYPT and Western blot with an specific phospho-antibody against Thr696. As shown in Figure 6C ISO increased the phosphorylation of MYPT at Thr696, which was prevented by preincubation with the ROCK inhibitor Y-27632, suggesting an involvement of the myosin phosphatase downstream ROCK.

PLD catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid, and choline and plays an im-

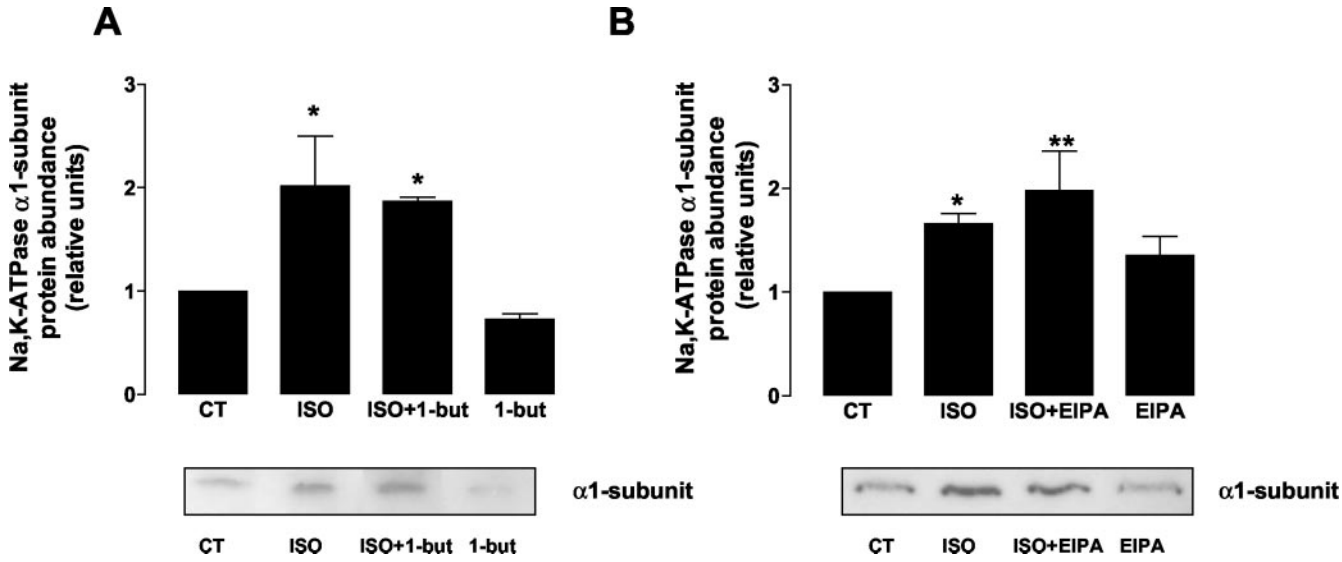

Figure 7. Role of Phospholipase D and Na^+-H^+ exchangers on ISO-induced Na,K-ATPase exocytosis. A549 cells were pretreated with (A) 1% 1-butanol, 15 min (to study PLD) and (B) 10 μM EIPA, 30 min (to study NHE), before incubating with 10 μM ISO for 15 min. Na,K-ATPase exocytosis was studied by biotin labeling of surface proteins followed by streptavidin pull-down and Western blot analysis using a specific antibody. Top: bars represent mean ± SEM (n = 3). *$p < 0.05$; **$p < 0.01$. Bottom: a representative Western blot.

portant role in regulated exocytosis. Primary alcohols are known to drive the generation of alcoholic phosphatidic acid from phosphatidylcholine, thus inhibiting the formation of phosphatidic acid itself (Jones *et al.*, 1999). Therefore, A549 cells were treated with 1% 1-butanol for 15 min, before stimulation with ISO. As shown in Figure 7A, Na,K-ATPase translocation from intracellular compartments to the plasma membrane was not inhibited by incubation with 1%-butanol nor with 1% ethanol (our unpublished results). These results suggest that PLD does not have a role in the ISO-mediated Na,K-ATPase exocytosis.

ISO-mediated Na,K-ATPase Exocytosis Was Independent of Na^+-H^+ Exchanger Regulation

One of the mechanisms by which the Na,K-ATPase function can be modulated is by the intracellular Na^+ concentrations ($[Na^+_i]$; Ewart and Klip, 1995). RhoA has been shown to regulate the Na^+-H^+ exchangers (NHE; Hooley *et al.*, 1996; Tominaga *et al.*, 1998). Therefore we conducted experiments to determine whether the effect of ISO on Na,K-ATPase was mediated by NHE stimulation. Thus, we incubated A549 cells with the amiloride analog EIPA (5-(*N*-ethyl-*N*-isopropyl) amiloride; 10 μM, 30 min) prior to stimulation with 10 μM ISO for 15 min and observed that EIPA did not prevent the effect of ISO on Na,K-ATPase exocytosis (Figure 7B). These data suggest that RhoA has a direct effect on the Na,K-ATPase exocytosis that is not due to NHE stimulation.

DISCUSSION

We present data supporting a role for the GTP-binding protein RhoA in the β-adrenergic receptor-mediated Na,K-ATPase exocytosis in AEC. We have identified components of the pathway leading to RhoA activation by β-adrenergic receptor stimulation and the downstream effectors involved in the Na,K-ATPase translocation from intracellular compartments to the plasma membrane resulting in increased Na,K-ATPase activity.

We have demonstrated, by studying translocation to the membrane fraction and pull-down with GST fusion proteins, that RhoA is activated by the β-adrenergic receptor agonist ISO in AEC. Also, we report that RhoA activation is necessary for Na,K-ATPase exocytosis. First, we utilized cholesterol-lowering agents (statins) that inhibit RhoA function by preventing its geranylgeranylation and membrane-targeting (Laufs *et al.*, 1999; Takeuchi *et al.*, 2000). Using this approach, we demonstrated that activation of RhoA is required in ISO-mediated Na,K-ATPase exocytosis. Second, we used permanently transfected A549 cells expressing dominant negative RhoA, an approach widely used to study the involvement of small GTP-binding proteins in different cellular events (Kjøller and Hall, 1999; see Figures 2 and 3), which also prevented the ISO-mediated exocytosis of the Na,K-ATPase. Taken together, these results suggest an important role for RhoA in the ISO-mediated increase in Na,K-ATPase activity by regulating the exocytosis of the Na,K-ATPase to the plasma membrane

Our data suggest that ISO, via β_2-adrenergic receptors, cAMP/PKA and G_i heterotrimeric G protein activates RhoA in AEC (see Figures 4 and 5). Classically, cAMP-dependent PKA activation results in phosphorylation of RhoA in its C-terminal region leading to its inactivation (Lang *et al.*, 1996). However, it has been reported that RhoA can be activated by ISO/cAMP (Yamauchi *et al.*, 2001), which is in agreement with our data. We reason that, G_s/cAMP-activated PKA does not phosphorylate RhoA directly; therefore, we studied whether another heterotrimeric G proteins could be involved in the ISO-mediated RhoA activation. β_2-adren-

ergic receptors have been shown to be coupled to the PTX-sensitive heterotrimeric G protein, G_i (Daaka *et al.,* 1997; Post *et al.,* 1999; Gosmanov *et al.,* 2002). To study G_i involvement we used two different strategies: incubation with PTX and AEC transfected with a minigene vector expressing $G\alpha_i$ COOH-terminal peptide that selectively blocks signal transduction through G_i proteins (Gilchrist *et al.,* 1999, 2002a, 2002b). We report that both G_s/cAMP/PKA and G_i are upstream of RhoA activation. Importantly, incubation with PTX also inhibited FSK-mediated RhoA stimulation (see Figure 5). These data lead us to reason, as recently reported (Lefkowitz, 1998), that receptor-dependent activation of G_s stimulates adenylyl cyclase, generates cAMP, and activates PKA, leading to phosphorylation of the β_2-adrenergic receptor, switching its coupling specificity from G_s to G_i. Then, G_i could regulate RhoA activation as previously reported (Laudanna *et al.,* 1996; Liu *et al.,* 2001). Traditionally, the heterotrimeric G proteins of the $G_{12/13}$ family have been reported to modulate RhoA activation (Buhl *et al.,* 1995; Fromm *et al.,* 1997; Hart *et al.,* 1998; Mao *et al.,* 1998). Therefore, to further study the role of heterotrimeric G proteins in the ISO-mediated RhoA activation, we used AEC transfected with minigene vectors expressing $G\alpha_{12}$ and $G\alpha_{13}$ COOH-terminal peptides, selectively blocking signal transduction through the $G_{12/13}$ family of heterotrimeric G proteins (Gilchrist *et al.,* 1999, 2002a, 2002b). Using this approach, we found that G_{12} and G_{13} are not involved in the stimulation of RhoA by β-adrenergic agonists (see Figure 5C).

As potential downstream effectors of RhoA, we focused on PLD and Rho-associated kinase. PLD is an important effector in receptor-mediated signal transduction pathways, and it has been described to play a role in the exocytosis of the GLUT4 glucose transporter in adipocytes (Emoto *et al.,* 2000; Bandyopadhyay *et al.,* 2001). However, PLD did not regulate the ISO-mediated Na,K-ATPase exocytosis (see Figure 7). In contrast, we provide evidence that ISO-mediated Na,K-ATPase exocytosis was prevented in A549 cells pretreated with the ROCK inhibitor Y27632 and in cells expressing dn-ROCK, suggesting that the pathway leading to the ISO-mediated Na,K-ATPase exocytosis occurs via RhoA/Rho-associated kinase (see Figure 6). Rho-associated kinase is known to play a role in the regulation of acto-myosin contraction (Kimura *et al.,* 1996; Maekawa *et al.,* 1999), and we found that the regulatory subunit of myosin phosphatase is phosphorylated by ROCK (i.e., inhibiting its activity). Thus, we propose that ROCK/MYPT may be important during Na,K-ATPase exocytosis because it has been reported that vesicle-associated myosin is capable of actin-based vesicular transport (Evans *et al.,* 1998; Lang *et al.,* 2000).

Na,K-ATPase regulation may be affected not only by translocation of Na,K-ATPase molecules from intracellular compartments to the plasma membrane, but also by changes in intracellular Na^+ concentrations (Bertorello and Katz, 1993; Ewart and Klip, 1995; Blanco and Mercer, 1998; Feraille and Doucet, 2001). NHE regulate intracellular Na^+ concentrations and several NHE isoforms have been shown to be modulated by RhoA (Tominaga and Barber, 1998; Szaszi *et al.,* 2000). Therefore, we studied whether a specific NHE inhibitor prevented ISO-mediated Na,K-ATPase exocytosis. We provide evidence that entry of Na^+ in the cell by NHE was not involved in the ISO-mediated Na,K-ATPase exocytosis, which suggests that RhoA has a direct effect on the Na,K-ATPase exocytosis rather that an indirect effect due to increased intracellular $[Na^+]_i$ concentration. This is in agreement with a previous report where the regulation of Na,K-ATPase by ISO was independent to the Na^+ entry via apical Na^+-channels (Bertorello *et al.,* 1999).

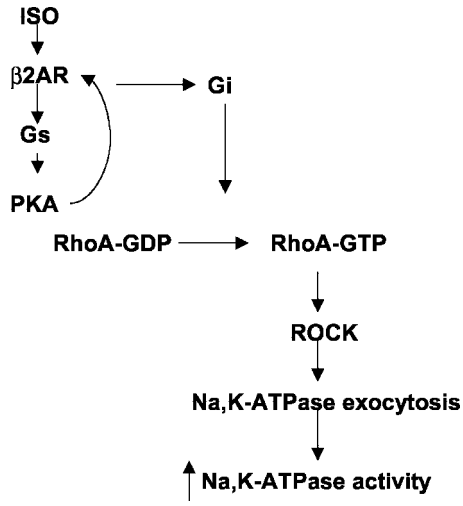

Figure 8. Proposed model of RhoA-mediated Na,K-ATPase exocytosis and increased catalytic activity by β-adrenergic agonists in AEC.

Reorganization of the actin cytoskeleton is a prerequisite for exocytosis, so as to enable docking and fusion of vesicles/secretory granules with the plasma membrane (Lang *et al.,* 2000). The role of RhoA in the exocytosis of proteins is cell type dependent. Several reports have described a role for RhoA in this process, such as in the stimulation of exocytosis of secretory granules in mast cells (Norman *et al.,* 1994, 1996; Sullivan *et al.,* 1999), exocytosis in pancreatic acini (Nozu *et al.,* 1999), and, as recently reported, in the translocation of the Na,K-ATPase itself in renal epithelial cells (Maeda *et al.,* 2002). However, other reports indicate an inhibitory role for RhoA, as reported in the translocation of aquaporin-2 induced by vasopressin in renal cells (Klussmann *et al.,* 2001).

In summary, we present here evidence for a positive role of RhoA/Rho-associated kinase in the β_2-adrenergic receptor-mediated Na,K-ATPase exocytosis and increased activity in AEC. As depicted in Figure 8, we propose a model for the pathway leading to the modulation of Na,K-ATPase activity by β_2-adrenergic receptor agonists. We propose that activation of β_2-adrenergic receptors leads to G_s/cAMP/PKA and G_i activation, which in turn activates RhoA and its downstream effector ROCK leading to the organization of the actin cytoskeleton necessary for Na,K-ATPase exocytosis and consequent increase in Na,K-ATPase activity.

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