cAMP-induced AQP2 translocation is associated with RhoA inhibition through RhoA phosphorylation and interaction with RhoGDI

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Summary

We have recently demonstrated that inhibition of Rho GTPase with *Clostridium difficile* toxin B, or with *Clostridium botulinum* C3 toxin, causes actin depolymerization and translocation of aquaporin 2 (AQP2) in renal CD8 cells in the absence of hormonal stimulation. Here we demonstrate that Rho inhibition is part of the signal transduction cascade activated by vasopressin leading to AQP2 insertion into the apical membrane. Quantitation of active RhoA (GTP-bound) by selective pull down experiments demonstrated that the amount of active RhoA decreased upon stimulation of CD8 cells with the cAMP-elevating agent forskolin. Consistent with this observation, forskolin treatment resulted in a decreased expression of membrane-associated (active) Rho, as assessed by cell fractionation followed by western blotting analysis. In addition, the abundance of the endogenous Rho GDP dissociation inhibitor (Rho-GDI) was found to have decreased in the membrane fraction after forskolin stimulation. Co-immunoprecipitation experiments revealed that, after forskolin stimulation, the amount of Rho-GDI complexed with RhoA increased, suggesting that Rho GTPase inhibition occurs through association of RhoA with Rho-GDI. Finally, forskolin stimulation was associated with an increase in Rho phosphorylation on a serine residue, a protein modification known to stabilize the inactive form of RhoA and to increase its interaction with Rho-GDI. Taken together, these data demonstrate that RhoA inhibition through Rho phosphorylation and interaction with Rho-GDI is a key event for cytoskeletal dynamics controlling cAMP-induced AQP2 translocation.

Key words: Aquaporin 2, RhoA, Rho-GDI, PKA, Actin cytoskeleton

Introduction

AQP2 is the vasopressin-regulated water channel involved in the modulation of water reabsorption in mammalian kidney. The peptide hormone vasopressin (AVP) acts by its interaction with V2 receptors, located on the basolateral membranes of collecting duct principal cells, inducing an increase in cAMP and subsequent activation of protein kinase A (PKA). This event results in the translocation of AQP2-bearing vesicles from an intracellular pool to the apical membrane (Klussmann et al., 2000; Knepper and Inoue, 1997).

G proteins are involved in the regulation of vesicle redistribution between intracellular compartments of the exocytic and endocytic pathways (Nuoffer and Balch, 1994), and subunits of Gi and Go proteins have been found associated with membranes in different cell lines. Heterotrimeric G proteins from the Gi family are required for cAMP-triggered trafficking of AQP2 (Valenti et al., 1998).

Furthermore, monomeric GTP-binding proteins from the Rab family were found in a kidney preparation enriched in AQP2-containing vesicles, and are likely involved in AQP2 intracellular membrane traffic (Liebenhoff and Rosenthal, 1995).

Depolymerization of cortical F-actin has been considered an important prerequisite for facilitation of exocytosis. Complex biological processes such as vesicle trafficking require precise control of actin cytoskeleton organization, which involves activation of the small GTP-binding protein Rho (Ren et al., 1999; Ridley et al., 1993). Rho is a member of the Ras superfamily of small GTP-binding proteins. Proteins from the Rho family (Cdc42, Rac1 and Rho) are involved in the regulation of actin polymerization. In particular, Cdc42 activates Rac1 and Rho proteins in serum-starved Swiss 3T3, and induces the formation of actin-based structures known as filopodia. The activation of Rac1 results in the formation of lamellipodia. In addition, Rho regulates the organization of stress fibers and the assembly of focal adhesion complexes (Nobes and Hall, 1995). GTP-binding proteins from the Rho family cycle between an active GTP-bound state and an inactive GDP-bound form. The exchange of hydrolyzed GDP for GTP results in a conformational change, unmasking structural domains by which they bind to the effectors. The activity of monomeric G proteins is controlled by different types of factors: GDP/GTP exchange protein (GEP) stimulates the interconversion between the GDP form and GTP form; and GTPase activating protein (GAP) binds to the GTP-form and stimulates the intrinsic GTPase activity of monomeric G-

proteins. GDP dissociation inhibitor (GDI) inhibits GDP dissociation, prevents GTP hydrolysis and maintains the Rho family members in a soluble form (Sasaki and Takai, 1998; Van Aelst and D'Souza-Schorey, 1997). The switch process for Rho family proteins presents an additional layer of regulation.

GTP-binding proteins from the Rho family are maintained associated with membrane fraction (active form) interacting with specific target molecules involved in the regulation of actin cytoskeleton. Translocation of Rho proteins from the membrane fraction to a soluble compartment correlates with their inactivation and with subsequent interaction with Rho-GDI. Recently, it was shown that RhoA is phosphorylated by PKA at Ser-188, and that this phosphorylation increases the interaction with GDP dissociation inhibitor (GDI), inhibiting the binding with downstream targets independently of the GTP/GDP state (Dong et al., 1998; Forget et al., 2002). This suggests that the cAMP signal mediates down-regulation of RhoA. Moreover, the cytosolic complex of the Rho GDP-bound form and Rho-GDI is not activated by Rho-GEP, suggesting the existence of another regulatory factor.

In renal collecting duct cells, we have recently demonstrated that inhibition of RhoA GTPase with *Clostridium difficile* toxin B and with *Clostridium botulinum* C3 toxin caused actin depolymerization and translocation of AQP2 to the plasma membrane in the absence of hormonal regulation (Klussmann et al., 2001; Tamma et al., 2001). The present work was undertaken to verify whether Rho inhibition is a physiological step in the signal transduction cascade activated by vasopressin and leading to AQP2 fusion with the apical membrane. To answer this question, we used a biochemical assay for the quantitation of Rho activity. Using this assay, we analyzed the contribution of soluble factors such as Rho-GDI to Rho activation/inactivation cycle in the signal transduction cascade initiated by elevation of cAMP and leading to AQP2 targeting to the plasma membrane. We also attempted to delineate other components of the Rho signaling pathway, notably the role of cAMP-dependent protein kinase A (PKA) phosphorylation of RhoA on a serine residue. We demonstrate that this event represents an additional layer of regulation of Rho activity in renal principal cells. The data reported here reveal several intriguing aspects of the regulatory role of Rho on AQP2 trafficking.

Materials and Methods

Materials and chemicals

The anti-RhoA and anti-Rho-GDI antibodies were purchased from Santa Cruz Biotechnology. Phosphoserine antibodies were from Sigma. ^{32}P orthophosphate was purchased from NEN (Life Science Products, Italy). Forskolin was purchased from Calbiochem. *C. difficile* toxin B was a kind gift from K. Aktories (Freiburg, Germany).

Cell culture

CD8 cells were established by stably transfecting the RC.SV3 rabbit cortical collecting duct cells with cDNA encoding rat AQP2 (Valenti et al., 1996). CD8 cells were grown at 37°C as described in a hormonally defined medium containing 5% newborn calf serum (Valenti et al., 1996). Confluent monolayers were used at days 3-5 after plating.

Expression and purification of GST-TRBD

The coding sequence for the Rhotekin Rho-binding domain (TRBD) cloned into the pGEX-2T vector (kindly provided by A. Schwartz, La Jolla, CA) was amplified in *E. coli*. *E. coli* expressing the fusion protein were inoculated in 1 liter of LB medium with ampicillin using a fresh bacterial preculture. Incubation was continued at 37°C until OD reached 0.5-0.6. Protein expression was then induced with 1 mM of isopropyl-b-D-thiogalactopyranoside (IPTG) for 30 minutes. The bacteria were collected by centrifugation at 5000 ***g*** and resuspended in PBS with protease inhibitors (3.2 mg/ml trypsin inhibitor, 1.4 mg/ml aprotinin, 0.5 mM benzamidine, 0.5 mM PMSF) before sonication. The bacteria were lysated for 30 minutes in PBS with 1% Triton X-100. The lysate was clarified by centrifugation at 17,000 ***g*** at 4°C for 10 minutes and the supernatant obtained was incubated with 450 μl of glutathione beads for 30 minutes at 4°C. The beads were washed three times at 4°C with PBS in the presence of protease inhibitors and used for affinity precipitation of cellular GTP-Rho.

Affinity precipitation of cellular GTP-Rho

Rho activity was evaluated in renal CD8 cells in three different experimental conditions: at rest, after elevation of cAMP levels with forskolin treatment and in Toxin-B-treated cells. Stimulation with forskolin was performed with 10^{-4} M forskolin for 15 minutes at 37°C. To evaluate the effect of toxin B on Rho activity, confluent monolayers were incubated in a cell culture medium containing toxin B (200 ng/ml) for 2 hours at 37°C. Cells were washed with ice-cold buffer containing 150 mM NaCl, 10 mM Tris-buffered pH 7.4 (TBS) and then lysed in ice-cold RIPA buffer containing 50 mM Tris-HCl pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 3.2 μg/μl trypsin inhibitor, 1.4 μg/μl aprotinin, 0.5 mM benzamidine, 0.5 mM PMSF. The cell lysate was clarified by centrifugation at 13,000 ***g*** for 5 minutes at 4°C and incubated with GST-RBD beads (20-30 μg) for 30 minutes at 4°C. The beads were washed three times with a buffer containing 50 mM Tris-buffered pH 7.2, 1% Triton, 150 mM NaCl, 10 mM $MgCl_2$, 3.2 μg/μl trypsin inhibitor, 1.4 μg/μl aprotinin, 0.5 mM benzamidine, 0.5 mM PMSF. GTP-Rho was eluted by boiling the precipitate in Laemmli buffer for 10 minutes in the presence of 40 mM DTT. Bound Rho proteins were detected by western blotting using a monoclonal antibody against RhoA. The densitometric analysis was performed using Scion Image Software for Windows. Statistical analysis was performed by one-way ANOVA and Tukey's multiple comparison test.

Membrane preparation

For the preparation of the fraction enriched in the plasma membranes (low speed pellet, LS) or in intracellular vesicles (high speed pellet, HS) control or forskolin-stimulated cells (10^{-4} M for 15 minutes at 37°C) were homogenized with a glass/Teflon homogenizer in ice-cold buffer containing 250 mM sucrose and 10 mM Tris pH 7.5. Cell suspensions were centrifuged at 700 ***g*** for 10 minutes at 4°C. The supernatant was centrifuged at 17,000 ***g*** for 45 minutes at 4°C. The resulting pellet (LS) enriched in plasma membrane was recovered in PBS and stored at –20°C. The supernatant was spun at 200,000 ***g*** in a Beckman Rotor 50 2Ti for 60 minutes at 4°C. The final pellet (HS) enriched in intracellular vesicles was recovered in PBS and stored at –20°C until used for immunoblotting studies.

Co-immunoprecipitation

CD8 cells were washed with PBS and homogenized with a glass Teflon homogenizer in lysis buffer containing 20 mM Tris-HCl, 1% Igepal, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5% deoxycholate, 0.1% SDS, 1.5 mM $MgCl_2$, 0.15 M NaCl, pH 8.0. The homogenates were centrifuged at 17,000 ***g***, 30 min at 4°C and the

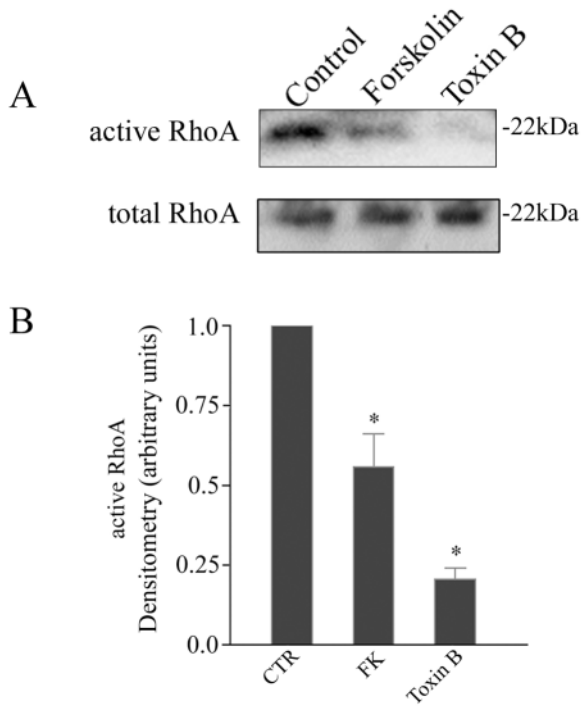

Fig. 1. Affinity precipitation of cellular GTP-Rho by pull-down assay. (A) CD8 cells were left untreated (control), stimulated with forskolin (10^{-4} M, 15 minutes) or incubated with *C. difficile* toxin B (200 ng/ml, 2 hours), which inhibits all proteins from the Rho family. The lysates from each condition were incubated with 20-30 μg of GST-RBD (Rho binding domain of rhotekin) conjugated with glutathione-Sepharose 4B beads. GTP-Rho was precipitated by centrifugation and subjected to western blotting analysis with anti-RhoA antibodies. Results are representative of three independent experiments with similar results. (B) Densitometric profile (mean values±s.e., n=3) of the GTP-RhoA band relative to control. *P<0.001.

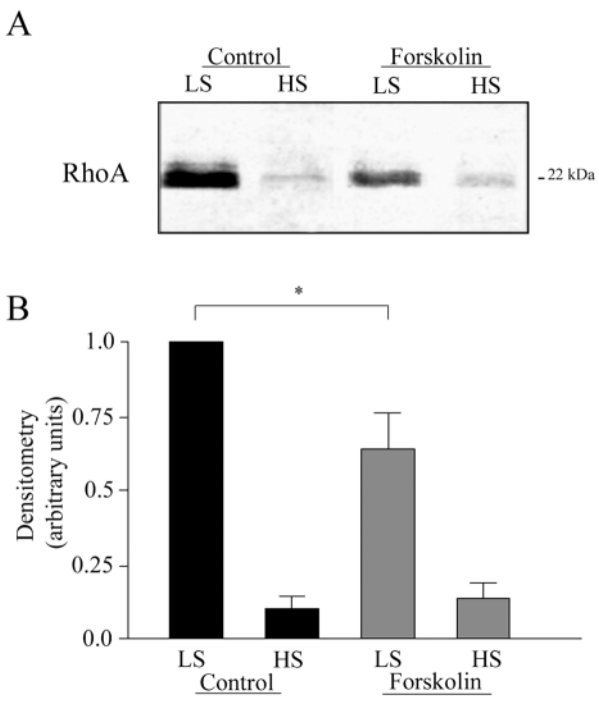

Fig. 2. Detection of RhoA in membrane fractions from CD8 cells. (A) Fractions enriched in plasma membrane (low speed pellet, LS) or in intracellular vesicles (high speed pellet, HS) were prepared from CD8 cells in basal condition and after forskolin stimulation. Equal amount of protein (30 μg/lane) were subjected to western blotting analysis (see above) with anti-RhoA-specific antibody (1:500) and revealed with alkaline-phosphatase-conjugated anti-mouse IgG (1:5000). (B) Densitometric analysis (mean values±s.e., n=4) of the RhoA 22 kDa band. *P<0.001.

supernatants were incubated overnight with 2 μg of anti-RhoA antibodies. Protein A-conjugated agarose beads (Sigma) were added and incubated for 2 h. The beads were washed three times with 1 ml of lysis buffer. Bound proteins were eluted with Laemmli sample buffer without DTT (95°C, 5 min) and subjected to Western blot analysis using Rho-GDI antibodies (Santa Cruz Biotechnology). Alternatively, the homogenates from control and forskolin-stimulated cells were incubated overnight with 30 μl agarose conjugated monoclonal anti-phosphoserine antibodies. Immunocomplexes, after washing, were eluted in Laemmli buffer, resolved in a 13% polyacrylamide gel and subjected to Western blot analysis for RhoA immunodetection.

Phosphorylation of RhoA in intact cells

Metabolic labeling of confluent CD8 cells with [^{32}P] orthophosphoric acid (NEN, Life Science Products, Italy, s.r.l.) was performed as previously described (Valenti et al., 2000). Briefly, confluent monolayers of CD8 cells, grown on 20 mm cell culture Petri dishes, were metabolically labeled with 250 μCi/ml of [^{32}P] orthophosphoric acid (NEN) in 0.5 ml of phosphate-free DMEM for 2 hours at 37°C in a 5% CO_2 atmosphere. CD8 cells were left untreated or stimulated with forskolin 10^{-4} M for 15 minutes at 37°C in PBS, with or without a 30 minute pretreatment with 30 μM H89. Cells were washed three times with PBS and quickly lysed in ice-cold immunoprecipitation buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM KCl, 5 mM EDTA, 2% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A. The lysate was clarified by centrifugation at 17,000 ***g*** for 10 minutes at 4°C and subjected to immunoprecipitation with 10 μl agarose-conjugated RhoA (Sigma, Immunochemicals). Immunocomplexes were washed three times in immunoprecipitation buffer, mixed with 30 μl of Laemmli buffer, heated at 95°C for 10 minutes and resolved in a 13% SDS-polyacrylamide gel. Gels were stained, dried and exposed to Kodak X-Omat AR film Lightning Plus intensifying screen at –80°C. To verify that identical amounts of RhoA were immunoprecipitated from each sample, gels were probed with anti-RhoA antibodies by western blotting.

SDS-polyacrylamide gel electrophoresis and immunoblot analysis

Proteins were resolved in a 13% polyacrylamide gel. Proteins were transferred onto Immobilon-P (Millipore) by standard procedures. Blots were incubated with primary antibody as reported in the figure legends and then for 45 minutes with a horseradish peroxidase-conjugated secondary antibody (1:3000 Sigma) and visualized using the ECL-Plus detection system (Amersham Life Science). Alternatively, blots were incubated with goat anti-mouse IgG alkaline-phosphatase-conjugated antibodies (1:5000 Sigma) and revealed for alkaline-phosphatase using 0.56 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.48 mM nitro blue tetrazolium in 10 mM Tris-HCl, pH 9.5 (Jansen, Pharmaceutica).

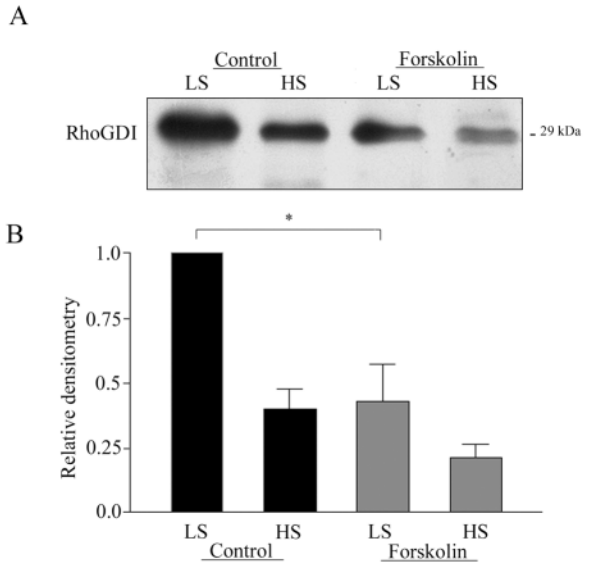

Fig. 3. (A) Detection of Rho-GDI in membrane fractions from CD8 cells. Membrane fractions enriched in plasma membrane (LS) or in intracellular vesicles (HS) were prepared from control and forskolin-stimulated CD8 cells. Equal amounts of protein (30 μg/lane) were separated by gel electrophoresis and immunoblotted with anti-Rho-GDI antibody (1:500). Results are representative of three independent experiments. (B) Densitometric profile (mean values±s.e., n=3) of the 29 kDa band. *P<0.001.

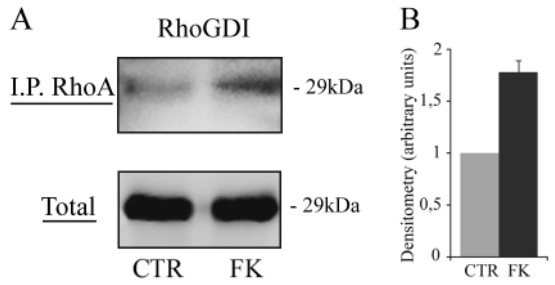

Fig. 4. Co-immunoprecipitation of RhoA and RhoGDI. (A) RhoA antibody was incubated with cell lysate from control and forskolin-stimulated CD8 cells. Immunocomplexes were precipitated with Protein-A-conjugated agarose. Proteins were eluted using Laemmli buffer and analyzed by immunoblot for detection of RhoGDI. This result is representative of three independent experiments demonstrating that forskolin stimulation is associated with the formation of a soluble RhoA-Rho-GDI complex. (B) Densitometric profile (mean values±s.e., n=3) of the 29 kDa band.

Results

Quantification of active Rho in CD8 cells by affinity-precipitation assay

We first determined the amounts of GTP-RhoA by affinity precipitation in CD8 cells. Rhotekin (a Rho effector) Rho-binding domain fused to glutathione S-Transferase (GST) was used to selectively pull down GTP-RhoA from cell lysates under different experimental conditions: basal, after stimulation with forskolin, or after pretreatment with B toxin. Equal amounts of total Rho were loaded on the gels (Fig. 1A, total Rho). Compared with control conditions, the amount of GTP-RhoA was strongly reduced after forskolin stimulation, consistent with an attenuation of Rho activity (Fig. 1A). As expected, a drastic reduction in GTP-bound cellular RhoA was observed in CD8 cells treated with toxin B, known to inactivate proteins of the Rho family [Fig. 1A (Boquet et al., 1998)]. Statistical analysis revealed that forskolin stimulation caused a 44±0.44% (n=3) attenuation of Rho activity, whereas an 80±0.04% (n=3) reduction in RhoA activity was obtained in toxin-B-pretreated cells (Fig. 1B).

The activities of the Rho family are regulated by several proteins that modulate their GTP/GDP state. The cycling between the two nucleotide-bound states is accompanied by cycling between the cytosolic fraction and the membranes (Mackay and Hall, 1998; Takaishi et al., 1995). To investigate whether Rho inhibition resulted in a decrease in membrane-associated Rho, cell fractionation of CD8 cells was performed from control and forskolin-stimulated cells. Cells were homogenized and the plasma membrane enriched fraction (LS) as well as the intracellular vesicles enriched fraction (HS) were obtained by differential centrifugation. Western blotting analysis of separated membrane fractions from control cells revealed that the majority of immunodetectable RhoA was associated with LS (Fig. 2A and densitometric analysis in Fig. 2B). After stimulation with forskolin, a significant decrease (36.3±0.13, n=4) in immunoreactive RhoA was observed in LS. This result indicates that the signal transduction pathway activated by forskolin and leading to AQP2 translocation involves inhibition of Rho GTPase with a concomitant dissociation from a membrane compartment.

Rho proteins cycle between active, GTP-bound and inactive GDP-bound states (Mackay and Hall, 1998). Rho-GDI appears to stabilize the inactive, GDP-bound form of the protein, forming a cytosolic complex with Rho-GDP (Bourmeyster and Vignais, 1996). CD8 cells were tested for the endogenous expression of Rho-GDI. Equal amounts (30 μg/lane) of proteins enriched in plasma membrane (LS) or in intracellular vesicles (HS) isolated from control or forskolin-stimulated CD8 cells were separated by gel electrophoresis and immunoblotted with specific anti-Rho-GDI antibody (Fig. 3A). Relative to HS, Rho-GDI appeared enriched in LS. After forskolin stimulation, the intensity of the band decreased in both LS and HS fractions (Fig. 3A and densitometry in Fig. 3B). This finding suggests that upon elevation of cAMP, Rho-GDI dissociates from the membranes. It has been reported that the GDP form of Rho is preferentially bound to Rho-GDI in a soluble complex (Chuang et al., 1993; Hart et al., 1992; Leonard et al., 1992; Sasaki et al., 1993). To investigate whether forskolin stimulation caused an increase in the molecular interaction between RhoA and RhoGDI, generating a soluble complex, co-immunoprecipitation experiments were performed in control and forskolin-stimulated CD8 cells. As shown in Fig. 4, the amount of RhoA-RhoGDI complex increased by 1.83±0.17-fold in forskolin-stimulated cells compared with control cells (Fig. 4A, I.P. RhoA and densitometry in Fig. 4B). This data confirmed that elevation of intracellular cAMP caused an increase in the molecular interaction between RhoA and RhoGDI.

It has been shown that RhoA phosphorylation at Ser188 by PKA stabilizes and increases the binding with Rho-GDI (Forget et al., 2002). Moreover, it has been suggested that following phosphorylation of RhoA by PKA, GTP-bound Rho

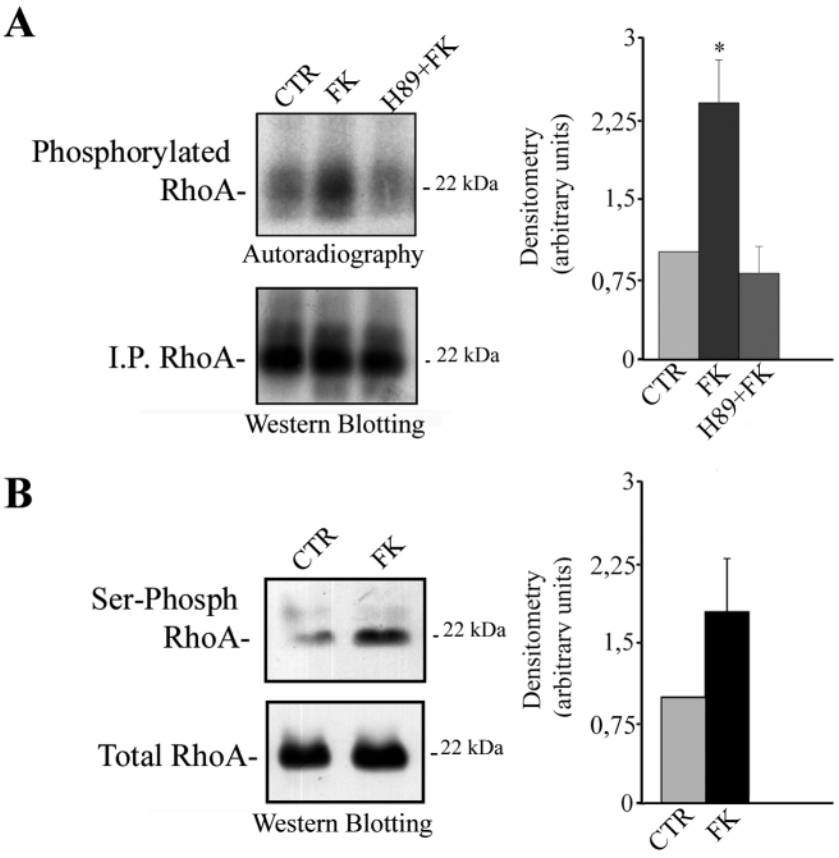

Fig. 5. Forskolin stimulation of CD8 cells results in RhoA phosphorylation at a serine residue. (A) For each experimental condition, confluent monolayers of CD8 cells were metabolically labeled with [^{32}P] orthophosphate (250 μCi/ml), for 3 hours. Cells were lysed and RhoA was immunoprecipitated with 10 μl agarose-conjugated RhoA and detected by autoradiography. Experimental conditions included: control condition, stimulation with forskolin (10^{-4} M for 15 minutes at 37°C); incubation with H89 (30 μM for 30 minutes at 37°C) followed by stimulation with forskolin. Equal amounts of RhoA were precipitated in each condition (I.P. RhoA). Forskolin stimulation increased the phosphorylated state of RhoA nearly 2.5-fold and pretreatment with H89 abolished the forskolin effect. A densitometric profile (mean values±s.e., n=3) of bands corresponding to phosphorylated RhoA is shown on the right. (B) Cell lysate from basal and forskolin-stimulated CD8 cells incubated with 30 μl monoclonal agarose conjugated anti-phosphoserine. Immunocomplexes were mixed with 20 μl of Laemmli buffer and blotted with anti-RhoA antibodies. Forskolin treatment resulted in a nearly two-fold increase in serine-phosphorylated RhoA. A densitometric profile (mean values±s.e., n=3) of bands corresponding to serine phosphorylated RhoA is shown on the right.

can be uncoupled from its putative effector independently of its binding to GTP or GDP (Lang et al., 1996). To investigate whether RhoA phosphorylation occurs during forskolin stimulation, CD8 cells were metabolically labeled with [^{32}P] orthophosphate, and either left untreated or stimulated with forskolin 10^{-4} M followed by RhoA immunoprecipitation. Equal amounts of immunoprecipitated RhoA were loaded on the gels and subjected to autoradiography. Forskolin stimulation caused a nearly 2.5-fold increase in RhoA phosphorylation, which was abolished in H89-pretreated cells, indicating that PKA is the kinase responsible for this effect (Fig. 5A and relative densitometric analysis). To confirm whether RhoA phosphorylation occurred at a serine residue, serine-phosphorylated proteins were immunoprecipitated from control and forskolin-stimulated cells, and RhoA was detected with specific anti-RhoA antibodies (see Materials and Methods for details) (Fig. 5B). Relative to the control, the amount of serine-phosphorylated RhoA immunoprecipitated from

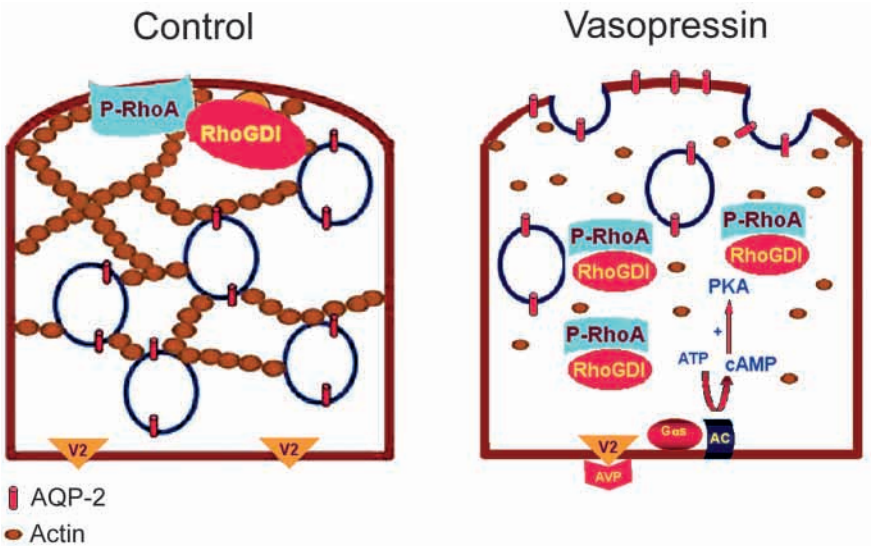

Fig. 6. Model depicting the role of RhoA in regulating actin cytoskeleton organization in vasopressin-stimulated renal cells. Vasopressin raises intracellular cAMP with consequent activation of PKA, which phosphorylates AQP2 and RhoA. Rho phosphorylation causes a decrease in the binding to its putative effectors, the Rho kinases. The attenuation of Rho activity results in depolymerization of F-actin, facilitating AQP2 insertion into the plasma membrane.

forskolin-stimulated cells was about twofold, demonstrating that the increase in phosphorylated RhoA involves a serine residue.

Discussion

We have previously demonstrated that treatment of CD8 cells and primary cultured IMCD cells with *Clostridium difficile* toxin B and with *Clostridium botulinum* C3 toxin, which inactivate Rho, caused actin depolymerization and translocation of AQP2 in CD8 cells in the absence of hormonal stimulation (Klussmann et al., 2001; Tamma et al., 2001). We show here that inhibition of Rho is a physiological event during forskolin stimulation in AQP2-transfected renal CD8 cells. Several types of evidence are reported in this study to support this conclusion. First, forskolin-triggered elevation of cAMP in CD8 cells resulted in a physiological attenuation of Rho activity, as assessed by quantitation of GTP-bound Rho (active) by pull-down experiments. This assay has been recently employed in different cell types, such as Swiss 3T3 cells (Ren et al., 1999) or smooth muscle cells (Sakurada et al., 2001) to measure Rho activity. In renal CD8 cells, this affinity-precipitation assay for endogenous GTP-bound (active) Rho allowed the detection of changes in Rho activity in vivo. These experiments demonstrated that forskolin stimulation caused a 44±0.44% attenuation of Rho activity. Second, forskolin treatment resulted in a decrease in membrane-associated (active) RhoA. Rho GTPases are synthesized as cytosolic proteins, but can associate with membranes by virtue of post-translational modifications, such as the prenylation of the CAAX motif present in the C-terminus (Clarke, 1992). Upon activation, Rho proteins have been shown to translocate from cytosol to membrane (Boivin and Beliveau, 1995; Fleming et al., 1996; Kranenburg et al., 1997; Philips et al., 1995). Therefore, the decrease in the amount of membrane-bound RhoA observed in response to the rise in cAMP levels, would confirm the inhibition of Rho activity in this physiological condition. Third, we show evidence that the amount of membrane-bound endogenous Rho-GDI also decreased after forskolin stimulation and, in parallel, the amount of Rho-GDI co-immunoprecipitated with RhoA increased. Rho-GDI belongs to the class of proteins shown to inhibit the release of GDP from Rho proteins (Fukumoto et al., 1990) and to solubilize membrane-associated Rho proteins (Isomura et al., 1991). Therefore, inhibition of Rho activity in response to cAMP is also accomplished by RhoA sequestration in the cytosol by a Rho-GDI-dependent mechanism.

Finally, the experiments reported here also include a novel finding, suggesting for the first time that the signal transduction pathway used by vasopressin causes PKA-dependent phosphorylation of RhoA, which might be an alternative pathway for terminating RhoA signaling in renal cells. We demonstrated that forskolin stimulation was associated with an increase in Rho phosphorylation on a serine residue, a protein modification known to stabilize the inactive form of RhoA and to increase its interaction with Rho-GDI (Lang et al., 1996). In fact, it has been reported that RhoA is phosphorylated by PKA at Ser188 and this phosphorylation increases the interaction with Rho-GDI, inhibiting the binding of downstream targets independently of the GTP/GDP state, indicating that the cAMP signal mediates downregulation of RhoA (Dong et al., 1998). Following phosphorylation of RhoA by PKA, GTP-bound Rho can be uncoupled from its putative effector independently of its binding to GTP or GDP. The emerging picture (Fig. 6) suggests that hormonal stimulation raises the level of intracellular cAMP and results in the activation of PKA which then phosphorylates AQP2 and RhoA, possibly at Ser188. Rho phosphorylation causes a decrease in the binding to its putative effectors, the Rho kinases (Dong et al., 1998). The attenuation of Rho activity would favor the depolymerization of F-actin, facilitating AQP2 insertion into the plasma membrane. Indeed, stimulation of CD8 cells with forskolin induces a depolymerization of F-actin-containing cytoskeletal structures (Valenti et al., 2000).

Taken together, these data demonstrate that RhoA inhibition through Rho phosphorylation and interaction with Rho-GDI is a key event for cytoskeletal dynamics controlling cAMP-induced AQP2 translocation.

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