An Inhibitory Role of Rho in the Vasopressin-mediated Translocation of Aquaporin-2 into Cell Membranes of Renal Principal Cells*

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Vasopressin regulates water reabsorption in renal collecting duct principal cells by a cAMP-dependent translocation of the water channel aquaporin-2 (AQP2) from intracellular vesicles into the cell membrane. In the present work primary cultured inner medullary collecting duct cells were used to study the role of the proteins of the Rho family in the translocation of AQP2. Clostridium difficile toxin B, which inhibits all members of the Rho family, Clostridium limosum C3 toxin, which activates only Rho, and the Rho kinase inhibitor, Y-27632, induced both depolymerization of actin stress fibers and AQP2 translocation in the absence of vasopressin. The data suggest an inhibitory role of Rho in this process, whereby constitutive membrane localization is prevented in resting cells. Expression of constitutively active RhoA induced formation of actin stress fibers and abolished AQP2 translocation in response to elevation of intracellular cAMP, confirming the inhibitory role of Rho. Cytochalasin D induced both depolymerization of the F-actin cytoskeleton and AQP2 translocation, indicating that depolymerization of F-actin is sufficient to induce AQP2 translocation. Thus Rho is likely to control the intracellular localization of AQP2 via regulation of the F-actin cytoskeleton.

The antidiuretic hormone arginine-vasopressin (AVP)1 regulates water reabsorption in renal collecting duct principal cells by inducing the translocation of the water channel aquaporin-2 (AQP2) from intracellular vesicles primarily into the apical cell membrane (shuttle hypothesis; Refs. 1 and 2). The molecular targets of AVP on the surface of principal cells are heptahelical vasopressin V2 receptors coupled to the Gα12/13 and adenyl cyclase system. Activation of this system by the hormone raises the level of intracellular cAMP and results in the activation of protein kinase A (PKA) which then phosphorylates its substrates, one of which is AQP2.

The phosphorylation of AQP2 by PKA and also the anchoring of PKA to subcellular compartments via protein kinase A anchoring proteins are prerequisites for AQP2 translocation to the cell membrane (2–5). In addition, the involvement of a heterotrimeric G protein of the Gαi family in the AQP2 translocation has been demonstrated in CD8 cells (6).

The cytoskeleton consists of various components, including microtubules and F-actin, both of which are involved in AVP-mediated changes of osmotic water permeability (2, 7–9). Microtubule-disrupting drugs like colchicine and nocodazole inhibit AVP-mediated increases in osmotic water permeability in renal collecting ducts by 65 and 72%, respectively (10–13). Disruption of the F-actin cytoskeleton by cytochalasin B or dihydrocytochalasin B inhibits the AVP-induced increase in osmotic water permeability in toad bladder epithelium by 25–50% (13, 14). The F-actin cytoskeleton also undergoes rearrangements after stimulation of cells with cAMP-elevating agents. After stimulation with vasopressin, total F-actin decreases in toad bladders by 20–30% (15) and apical F-actin in rat collecting duct principal cells by 26% (16). In CD8 cells, F-actin decreases in response to forskolin (17). The mechanisms underlying these changes of the F-actin cytoskeleton are not understood, and their influence on the fusion of AQP2-bearing vesicles with the cell membrane are not known. Valent et al. (17) recently suggested that the rearrangement of apical F-actin might be a prerequisite for promoting redistribution of AQP2-containing vesicles and a consequence of phosphorylation and dephosphorylation processes, mediated by PKA and protein phosphatases 1 and 2A, respectively.

Proteins of the Rho family (Rho, Rac, and Cdc42) of small GTP-binding proteins are active in their GTP-bound and inactive in their GDP-bound forms. They participate through their effectors in the organization of the actin cytoskeleton. As initially shown in fibroblasts, activation of Rho triggers the assembly of actin stress fibers and formation of focal adhesions, activation of Rac leads to formation of lamellipodia and membrane ruffles, and activation of Cdc42 induces surface protrusions designated filopodia (18–20). In addition, Rho family proteins are involved in the regulation of vesicle motility (21), endocytic events like receptor-mediated endocytosis (22, 23) and various exocytic processes. For example, the inactivation of Cdc42 and Rac by Clostridium sordellii lethal toxin inhibits the Ca2+-triggered release of hexoseaminidase from rat baso-
philic leukemia cells (RBL 2H3 cells; Ref. 24). This finding was confirmed by expression of dominant negative forms of Cdc42 and Rac in these cells (25). Inhibition of Rho by Clostridium botulinum C3 toxin prevented Ca\(^{2+}\)-triggered release of hexoseaminidase in permeabilized mast cells by 80% (26). Conversely, constitutively active RhoA or Rac1 greatly enhanced Ca\(^{2+}\)-induced secretion from permeabilized mast cells (27).

These findings led to the hypothesis that Rho proteins may be involved in the control of AQP2 translocation to the cell membrane. A recently described primary cell culture model of rat IMCD cells (28) was utilized to test this hypothesis. The cells were incubated with Clostridium difficile toxin B, which inhibits all members of the Rho family by glucosylation (Rho at Thr\(^35\), Rac and Cdc42 at Thr\(^35\), Ref. 29), or microinjected with Clostridium limosum-derived C3-fusion toxin, which inactivates Rho by ADP-ribosylation at Asn\(^41\) but affects neither Rac nor Cdc42 (30). In addition, IMCD cells were incubated with the Rho kinase inhibitor, Y-27632, and microinjected with a constitutively active mutant of RhoA. Surprisingly our data suggest tonic inhibition of AQP2 translocation by Rho, which is relieved by an increase in cAMP.

**EXPERIMENTAL PROCEDURES**

**Toxins, Phalloidin, Antibodies, and Plasmids—C. difficile toxin B** (29) and C. limosum-derived C3-fusion toxin consisting of full-length C. limosum C3 toxin fused to the (inactive) N-terminal part of the active-ADP-ribosylating C2I component of the C. botulinum binary toxin C2 (C3-fusion toxin) were prepared as described before (30). Y-27632 was kindly provided by Welfide Corp. (Osaka, Japan; Refs. 31 and 32).

TRITC-conjugated phalloidin was purchased from Sigma (Deisenhofen, Germany) and Oregon green coupled to dextran from Molecular Probes (Leiden, Netherlands). AQP2 was detected with a polyclonal antiserum raised against the C terminus of rat AQP2 (28, 33). Anti-rabbit Cy3-conjugated antibodies were purchased from Dianova (Hamburg, Germany).

A plasmid (pEXV-Myc-V14-RhoA) encoding constitutively active human RhoA (RhoA-V14) was kindly provided by A. Hall (34). The insert encoding RhoA-V14 was excised from the plasmid using the restriction enzymes BamHI and EcoRI, and subcloned into the BglII and EcoRI restriction sites of the plasmid pEGFP-C1 (CLONTECH, Heidelberg, Germany) to construct a plasmid encoding a fusion protein (RhoA-V14-GFP) consisting of RhoA-V14 and the green fluorescent protein (GFP).

**Culture of IMCD Cells—** Rat renal inner medullary cells were the source of primary cultured IMCD cells (28). Experiments were performed 6 days after seeding. Dibutyryl cAMP (Bt2cAMP), present in the culture medium for maintenance of AQP2 expression, was removed 16 h prior to experiments with the exception of the experiments in which the influence of hourly vasopressin (AVP) on AQP2 translocation was determined.

**Microinjection of C3-fusion Toxin and Plasmids into IMCD Cells—** Microinjection was performed as described (35). In brief, IMCD cells were seeded at a density of 7 × 10\(^4\) per cm\(^2\) on type IV collagen-coated coverslips with grids. C3-fusion toxin (40 µg/ml; Ref. 30) was dissolved in phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 7.8 mM NaH\(_2\)PO\(_4\), 1.4 mM KH\(_2\)PO\(_4\), pH 7.3, adjusted to 290 mosmol/kg with 1 mM NaCl) and co-microinjected with a plasmid encoding a fusion protein (RhoA-V14-GFP) consisting of RhoA-V14 and the green fluorescent protein (GFP). The Effect of Bacterial Toxins and the Rho Kinase Inhibitor, Y-27632, on the F-actin Cytoskeleton of IMCD Cells—Prior to investigating the role of GTP-binding proteins of the Rho family in the translocation of AQP2, bacterial toxins which affect the activity of these proteins were tested for their ability to alter F-actin-containing cytoskeletal structures, i.e. actin stress fibers (Fig. 1). For this purpose IMCD cells were incubated with toxin B (4 µg/ml) for 3.5 h. C3-fusion toxin was microinjected (40 µg/ml) and the cells were prepared for detection of F-actin 20 min after microinjection. Cells were co-microinjected with Oregon green coupled to dextran (2.5 mg/ml). In addition, cells were incubated with Y-27632 (100 µM, 1 h). After these treatments the cells were fixed, permeabilized, and F-actin was detected by incubation with TRITC-conjugated phalloidin (0.1 mg/ml, 30 min). F-actin was subsequently visualized by epifluorescence microscopy (Fig. 1).

The Effect of Bacterial Toxins and the Rho Kinase Inhibitor, Y-27632, on the F-actin Cytoskeleton of IMCD Cells—Prior to investigating the role of GTP-binding proteins of the Rho family in the translocation of AQP2, bacterial toxins which affect the activity of these proteins were tested for their ability to alter F-actin-containing cytoskeletal structures, i.e. actin stress fibers (Fig. 1). For this purpose IMCD cells were incubated with toxin B (4 µg/ml) for 3.5 h. C3-fusion toxin was microinjected (40 µg/ml) and the cells were prepared for detection of F-actin 20 min after microinjection. Cells were co-microinjected with Oregon green coupled to dextran (2.5 mg/ml). In addition, cells were incubated with Y-27632 (100 µM, 1 h). After these treatments the cells were fixed, permeabilized, and F-actin was detected by incubation with TRITC-conjugated phalloidin (0.1 mg/ml, 30 min). F-actin was subsequently visualized by epifluorescence microscopy (Fig. 1).

**Stimulation of IMCD cells with AVP (100 nM, 1 h)** induced a slight decrease in F-actin compared with non-stimulated control cells (Fig. 1). A detailed analysis by optical sections of 1 µm through the cells indicated a decrease mainly of the cortical F-actin (data not shown; Ref. 16). In toxin B-, C3-fusion toxin-, and Y-27632-treated IMCD cells, the phalloidin staining was strongly reduced, indicating depolymerization of F-actin. At later time points (toxin B > 4 h; C3-fusion toxin > 1 h), the cells rounded up and detached from the surface of the culture dish (data not shown). Incubation with toxin B, Y-27632, or microinjection of C3-fusion toxin resulted in an apparently stronger staining of the cortical F-actin. This impression may be due to the disappearance of intracellular F-actin, blunting visualization of cortical F-actin in untreated cells.

These data show that the toxins in the concentrations applied are effective in altering F-actin-containing structures, i.e. the actin stress fibers. Within the time frame indicated, shape changes were not detected in the majority of cells. Therefore these concentrations of the toxins were applied in subsequent experiments.

**Inhibition of Proteins of the Rho Family by Toxin B Induces Translocation of AQP2 into IMCD Cell Membranes in the Absence of Vasopressin—** Having established the effectiveness of toxin B in modulating F-actin-containing structures, the toxin was applied to investigate whether proteins of the Rho family are involved in the regulation of AQP2 translocation in IMCD cells (Fig. 2). Incubations with toxin B were carried out as described above. The distribution of AQP2 was determined by laser scanning immunofluorescence microscopic analyses using specific antisera. Fig. 2 shows a mainly intracellular distribution of AQP2 in untreated IMCD cells (control). After stimulation of the cells with AVP, AQP2 staining was mainly observed at the basolateral cell membrane as shown by both xy and z scans (see also: Refs. 4 and 28). Incubation of the cells with toxin B alone was sufficient to invoke a strong redistribution of AQP2 (Fig. 2). Addition of AVP (100 nM) 1 h prior to the cessation of toxin B treatment resulted in a further translocation of AQP2 (Fig. 2), as is evident from the further decrease in intracellular fluorescence.
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To quantify the effects of AVP and toxin B, intracellular and cell membrane fluorescence signal intensities were related to nuclear fluorescence signal intensities by analysis of laser scanning microscopic images, and the ratios of intracellular/ cell membrane fluorescence signal intensities were determined (Fig. 3; Ref. 4). A ratio >1, indicating a predominant intracellular localization of AQP2, was obtained for control cells (1.75 ± 0.06; mean ± S.E.). Ratios <1, statistically different from untreated control cells, were obtained for AVP-stimulated cells (0.53 ± 0.03) and for cells incubated with toxin B in the absence of AVP (0.70 ± 0.01), indicating a predominant localization of AQP2 at the cell membrane. These results suggest an inhibitory role of Rho proteins, whereby constitutive membrane localization of AQP2 is prevented in resting IMCD cells. The ratio of intracellular/cell membrane fluorescence signal intensities after stimulation of toxin B-pretreated cells was 0.54 ± 0.02. This ratio was significantly different from both untreated control cells and toxin B-pretreated cells.

**Activated Rho Abolishes cAMP-mediated Translocation of AQP2 into IMCD Cell Membranes**—The effects of Rho inactivation described above prompted us to study the effect of constitutively active Rho on AQP2 redistribution in response to elevation of intracellular cAMP. For this purpose, IMCD cells were microinjected with a plasmid encoding a fusion protein (RhoA-V14-GFP) of constitutively active RhoA (RhoA-V14) and the green fluorescent protein (GFP; 100–200 copies/cell). RhoA-V14-expressing cells were visualized by the detection of GFP fluorescence. The activity of RhoA-V14 was assayed by its ability to induce actin stress fiber formation 3 h after microinjection of the cells (Fig. 4).

**Depolymerization of the F-actin Cytoskeleton by Cytochalasin D Induces Translocation of AQP2 into IMCD Cell Membranes**—The data presented above show the involvement of Rho in the translocation of AQP2. To test whether this proceeds independently of the F-actin cytoskeleton or whether this is possibly mediated by Rho via the regulation of the F-actin-containing network, F-actin was depolymerized by incubation of the cells with cytochalasin D (2 μM) for 6, 11, 16, 25, and 30 min. This treatment induced a gradual decrease in F-actin content over 0–25 min (Fig. 6). After 16 min, a pronounced F-actin depolymerization was induced, and cell shape changes were observed. At 25 min, the cortical F-actin appeared thickened and

**Inhibition of Rho or Rho Kinases Induces Redistribution of AQP2 into IMCD Cell Membranes**—To identify the protein of the Rho family involved in the translocation of AQP2, C3-fusion toxin, which specifically inactivates Rho, was used (see "Experimental Procedures"; Ref. 30). The toxin was microinjected into the cytosol together with Oregon green coupled to dextran. The dye was employed for identification of microinjected cells and had no effect on the intracellular localization of AQP2 (data not shown). Non-injected control cells surrounding the microinjected ones show a predominantly intracellular localization of AQP2 (Fig. 2). In microinjected cells, however, AQP2 staining at the cell membrane is considerably increased, indicating a redistribution of AQP2. Likewise, inhibition of the downstream effectors of Rho, the Rho kinases, by Y-27632 (31, 32) induced a translocation of AQP2 to the cell membrane (Fig. 2).

Quantitative analysis of the effect of C3-fusion toxin and Y-27632 on IMCD cells (Fig. 3) yielded ratios of intracellular/ cell membrane fluorescence signal intensities of 0.94 ± 0.04 and 0.77 ± 0.05, respectively, which were significantly different from those obtained for control cells. These data further support the notion that a Rho-dependent pathway prevents a constitutive membrane localization of AQP2 in resting IMCD cells.

**Toxic B-pretreatment of AQP2**—Toxic B-pretreatment of AQP2 (40 μg/ml) was microinjected into the cytosol together with Oregon green coupled to dextran. The dye was employed for identification of microinjected cells and had no effect on the intracellular localization of AQP2 (data not shown). Non-injected control cells surrounding the microinjected ones show a predominantly intracellular localization of AQP2 (Fig. 2). In microinjected cells, however, AQP2 staining at the cell membrane is considerably increased, indicating a redistribution of AQP2. Likewise, inhibition of the downstream effectors of Rho, the Rho kinases, by Y-27632 (31, 32) induced a translocation of AQP2 to the cell membrane (Fig. 2).

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the cells were clearly shrunken. The apparently stronger staining of the cortical F-actin may be due to the disappearance of intracellular F-actin which in the untreated control cells blunts visualization of cortical F-actin. After 30 min, F-actin acquired a punctate appearance, and holes in the monolayer appeared.

were fixed, permeabilized, and incubated with anti-AQP2 and secondary cy3-conjugated anti-rabbit antibodies. AQP2 immunofluorescence was detected by laser scanning microscopy. The upper part of each panel represents xy scans; the lower part represents xz scans along the white line in the xy image. Scale bars, 20 μm.

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**FIG. 2.** Localization of AQP2 in IMCD cells. IMCD cells were either left untreated (control), incubated with AVP (100 nM, 1 h), toxin B (4 μg/ml, 3.5 h), or Y-27632 (100 μM, 1 h). C3-fusion toxin (40 μg/ml, 20 min) was microinjected. Microinjected cells were visualized by coinjected Oregon green coupled to dextran (2.5 mg/ml). AVP (100 nM) was added to toxin B-pretreated cells 1 h prior to cessation of toxin treatment. After incubations for the indicated lengths of time, the cells were fixed, permeabilized, and incubated with anti-AQP2 and secondary cy3-conjugated anti-rabbit antibodies. AQP2 immunofluorescence was detected by laser scanning microscopy. The upper part of each panel represents xy scans; the lower part represents xz scans along the white line in the xy image. Scale bars, 20 μm.

**FIG. 3.** Quantitative analysis of the effects of bacterial toxins, Y-27632, cytochalasin D and RhoA-V14 on the localization of AQP2 in IMCD cells. IMCD cells were treated as indicated in Figs. 1, 2, and 4–7. Immunofluorescence signals were detected by laser scanning microscopy (4). The intracellular and cell membrane immunofluorescence signal intensities were determined and related to nuclear signal intensities (n = 25 for untreated control, n = 14 for + AVP, n = 41 for toxin B – AVP, n = 24 for toxin B + AVP, n = 27 for C3-fusion toxin, n = 24 for Y-27632, n = 9 for RhoA-V14, n = 15 for cytochalasin D for 6 and 11 min, n = 12 for cytochalasin D for 16 min; mean ± S.E.; three independent experiments). The ratios of intracellular/cell membrane fluorescence signal intensities were calculated. Ratios > 1 indicate a predominantly intracellular localization of AQP2 and ratios < 1 a predominant localization at the cell membrane. Values significantly different from untreated control cells are indicated (asterisks, p < 0.001).

**FIG. 4.** The effect of the expression of a constitutively active RhoA mutant (RhoA-V14) on the F-actin cytoskeleton in IMCD cells. IMCD cells were cultured in the presence of Bt2cAMP and microinjected with a plasmid (100–200 copies/cell) encoding RhoA-V14 fused to the GFP. RhoA-V14 expression was analyzed 3 h after microinjection by detection of GFP fluorescence (epifluorescence microscopy; RhoA-V14-GFP; left panel). Thereafter, the cells were fixed, permeabilized, and incubated with TRITC-conjugated phalloidin (right panel) to visualize the F-actin cytoskeleton (see legend to Fig. 1). TRITC fluorescence was detected by epifluorescence microscopy. Scale bars, 20 μm.

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At early time points, i.e. when cytochalasin D-mediated F-actin depolymerization had commenced (6–11 min), AQP2 translocated to the cell membrane (Fig. 7). The ratios of intracellular/membrane signal intensities were 0.87 $\pm$ 0.03 at 6 min and 0.67 $\pm$ 0.05 at 11 min, indicating a continuing translocation of AQP2 to the cell membrane (Fig. 3). The intracellular staining of AQP2 after 16 min of incubation with cytochalasin D appeared stronger compared with that at 11 min (Fig. 7); the ratio of intracellular/cell membrane signal intensities was 0.84 $\pm$ 0.05 (Fig. 3). As described above, longer incubation with cytochalasin D (25–30 min) severely affected the IMCD cells even to the point of cell death. Therefore no conclusive data could be obtained regarding the intracellular localization of AQP2 at these time points.

These results clearly indicate that partial depolymerization of F-actin by cytochalasin D is sufficient to induce translocation of AQP2 to the cell membrane. These data are consistent with the hypothesis that Rho influences AQP2 translocation via regulation of F-actin-containing cytoskeletal structures.

DISCUSSION

Previous studies using toad bladders have demonstrated the involvement of the F-actin cytoskeleton in AVP-mediated increases in osmotic water permeability (13, 14). In addition, the reorganization of the F-actin cytoskeleton by AVP is a consistent finding in both amphibian urinary bladder epithelia and mammalian renal principal cells (2, 16). The small GTP-binding proteins of the Rho family (Rho, Rac, and Cdc42) are involved in the regulation of the F-actin cytoskeleton. The aim of this study was to investigate their possible involvement in the translocation of AQP2 to the cell membrane. Inactivation of all members of the proteins of the Rho family by incubation of IMCD cells with toxin B or selective inactivation of Rho by C3-fusion toxin induced the translocation of AQP2 in the absence of AVP. In addition, inhibition of effectors of Rho, the Rho kinases, induced a translocation of AQP2 to the cell membrane to a similar extent as C3-fusion toxin in the absence of AVP. Conversely, expression of a constitutively active mutant of RhoA (RhoA-V14) abolished the cAMP-mediated translocation of AQP2. These findings indicate that Rho plays an inhibitory role in the AQP2 shuttle. The inhibitory role of Rho in a cAMP-triggered exocytotic event (AQP2 translocation) is in contrast to the stimulatory role of Rho in Ca$^{2+}$-triggered exocytosis in RBL 2H3 and mast cells (see Introduction).

The inhibitory role of Rho in a cAMP-triggered exocytotic event was confirmed using the permanent rabbit cortical collecting duct cell line CD8, stably transfected with rat AQP2. These cells respond to forskolin by translocation of AQP2 into the apical cell membrane (6, 17, 36). Inhibition of Rho induced the translocation of AQP2 to the membrane of resting CD8 cells

Fig. 5. Localization of AQP2 in IMCD cells expressing a constitutively active mutant of RhoA (RhoA-V14). IMCD cells were cultured in the presence of Bt$_2$cAMP and microinjected with a plasmid encoding RhoA-V14 fused to the GFP (see Fig. 4). RhoA-V14 expression was analyzed 3 h after microinjection by detection of GFP fluorescence (epifluorescence microscopy; RhoA-V14-GFP; left panel). Thereafter, the cells were fixed, permeabilized, incubated with anti-AQP2 and secondary cy3-conjugated anti-rabbit antibodies. AQP2 immunofluorescence was detected by epifluorescence microscopy. The overlay of GFP and AQP2 fluorescence signals is shown in the right panel. Scale bars, 20 $\mu$m.

0 min

6 min

11 min

16 min

25 min

30 min

Fig. 6. The effect of cytochalasin D on the F-actin cytoskeleton in IMCD cells. IMCD cells were left untreated (0 min) or incubated with cytochalasin D (2 $\mu$m) which induces depolymerization of the F-actin cytoskeleton. At the time points indicated, the cells were fixed, permeabilized, and incubated with TRITC-conjugated phalloidin (0.1 mg/ml) to visualize the F-actin cytoskeleton (see legend to Fig. 1). TRITC fluorescence was detected by epifluorescence microscopy. Scale bars, 20 $\mu$m.

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Induced an incomplete translocation of AQP2. This effect may be explained by a partial inactivation of the inhibitory Rho family-dependent signaling pathway. This assumption is supported by the observation that longer incubations with the toxins (toxin B > 4 h; C3-fusion toxin > 1 h) induced cell shape changes and detachment of the cells from the culture dishes, indicating that the Rho family-dependent pathway is not completely inhibited by the toxins at earlier time points.

In IMCD cells, toxin B caused a 2-fold increase in osmotic water permeability ($P_f$). This was shown by applying a new technique using laser scanning reflection microscopy, established for determining osmotic water permeability coefficient changes in single adherent cells (37). These data confirm the inhibitory role of the proteins of the Rho family in the translocation of AQP2 and show that this inhibition is functionally relevant.

Incubation of IMCD cells with toxin B or Y-27632 and microinjection of C3-fusion toxin resulted not only in AQP2 translocation but also in depolymerization of F-actin containing stress fibers (Fig. 1). Similarly, cytochalasin D caused both depolymerization of the F-actin cytoskeleton and translocation of AQP2 within 6–11 min of incubation (Figs. 6 and 7). Thus depolymerization of the F-actin cytoskeleton is sufficient to induce the translocation of AQP2, and the F-actin cytoskeleton apparently prevents translocation of AQP2-bearing vesicles to the cell membrane of resting cells. Its depolymerization allows AQP2-bearing vesicles to translocate to the cell membrane. The molecular basis of this observation may be explained by different hypotheses. It is feasible that AQP2-bearing vesicles are linked directly to the F-actin cytoskeleton as postulated by Brown et al. (9). Alternatively an intermediate protein may be required, as postulated by Umenishi et al. (38).

There are a few cell types in which the disassembly of the F-actin cytoskeleton can trigger exocytosis. However, the role of Rho has not been investigated in this context. In pancreatic acinar cells, the introduction of the actin monomer-binding protein β-thymosin, which induces limited F-actin depolymerization, into permeabilized cells, results in rapid amylase release without applying additional stimuli (39). In alveolar epithelial type II cells (AET2 cells), C2 toxin, which depolymerizes the F-actin cytoskeleton, increased basal surfactant secretion (40).

The results presented here may be explained by the following model: stimulation of IMCD cells with AVP results in an increase in intracellular cAMP, which in turn activates PKA. One of the substrates of PKA is Rho, which is phosphorylated at Ser188 (41). The phosphorylation of Rho decreases the binding of RhoA to Rho kinases (42). The subsequent attenuation of Rho activity would favor depolymerization of the F-actin cytoskeleton and allow translocation of AQP2 into the cell membrane. Consistent with this model, our results show that stimulation of IMCD cells with AVP induces a depolymerization of the F-actin cytoskeleton (Fig. 1). Induction of polymerization of F-actin-containing stress fibers by expression of constitutively active RhoA (RhoA-V14) abolished the translocation of AQP2 in response to continuous stimulation of IMCD cells with Bt2cAMP (Fig. 5). In addition, incubation of IMCD cells with cytotoxic necrotizing factor I, derived from pathogenic Escherichia coli, which activates all members of the Rho family by deamidation (29), strongly induced the formation of actin stress fibers and inhibited AVP-mediated AQP2 translocation (data not shown). The increase in F-actin content may either increase the binding of AQP2-bearing vesicles to the F-actin cytoskeleton or the F-actin cytoskeleton might become a barrier for these vesicles similar to the barrier function of cortical F-actin in other exocytic processes. For example, in

**Fig. 7.** The effect of cytochalasin D on the localization of AQP2 in IMCD cells. IMCD cells were incubated without (control) or with AVP (100 nM, 1 h) or cytochalasin D (2 μM) for the indicated lengths of time. After incubations, cells were fixed, permeabilized, incubated with anti-AQP2 and secondary Cy3-conjugated anti-rabbit antibodies. AQP2 immunofluorescence was visualized by laser scanning microscopy. The upper part of each panel represents xy scans; the lower part represents xz scans along the white line in the xy image. Scale bars, 20 μm.

and expression of constitutively active Rho prevented the cAMP-triggered translocation.

Compared with AVP, toxin B, C3-fusion toxin, and Y-27632
chromaffin cells, pancreatic acinar cells and mast cells, cortical F-actin disassembly is considered a prerequisite for exocytosis (39, 43–45). The model proposed above is further supported by the finding that RhoA and RhoB are present in particulate fractions prepared from non-stimulated IMCD cells (data not shown). Since activation of Rho is accompanied by a translocation from the cytosol to membranes, this finding may indicate that RhoA and RhoB are either active or in the process of activation (46–48).

The only other system in which Rho appears to inhibit exocytosis via F-actin is the bovine chromaffin cell (45). Gasman et al. (49, 50) described a vesicle-associated signaling cascade in resting chromaffin cells in which the heterotrimeric G protein $G_{q}$ activates phosphatidylinositol 4-kinase via Rho. Activation of phosphatidylinositol 4-kinase may stabilize F-actin, thereby excluding secretory vesicles from exocytosis. For two reasons it is tempting to speculate that a similar mechanism underlies the inhibition of the AQP2 translocation: (i) mastoparan-sensitive heterotrimeric G proteins of the $G_{q}$ family have been co-purified with AQP2-bearing vesicles from CD8 cells (6), and, (ii) the finding that Rho proteins are present in IMCD and CD8 cell fractions enriched for AQP2-bearing vesicles (data not shown). Final proof that Rho and phosphatidylinositol 4-kinase reside on AQP2-bearing vesicles will require analysis of the protein pattern of immuno-isolated vesicles using AQP2-, Rho-, and phosphatidylinositol 4-kinase-specific antibodies. In summary, the data presented here show that active Rho prevents AQP2 translocation from intracellular vesicles to the cell membrane, presumably by stimulating the formation of the F-actin cytoskeleton.

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