The prostaglandin E_2 analogue sulprostone antagonizes vasopressin-induced antidiuresis through activation of Rho

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Summary

Arginine-vasopressin (AVP) facilitates water reabsorption in renal collecting duct principal cells by activation of vasopressin V2 receptors and the subsequent translocation of water channels (aquaporin-2, AQP2) from intracellular vesicles into the plasma membrane. Prostaglandin E_2 (PGE_2) antagonizes AVP-induced water reabsorption; the signaling pathway underlying the diuretic response is not known. Using primary rat inner medullary collecting duct (IMCD) cells, we show that stimulation of prostaglandin EP_3 receptors induced Rho activation and actin polymerization in resting IMCD cells, but did not modify the intracellular localization of AQP2. However, AVP-, dibutyryl cAMP- and forskolin-induced AQP2 translocation was strongly inhibited. This inhibitory effect was independent of increases in cAMP and cytosolic Ca^{2+}. In addition, stimulation of EP_3 receptors inhibited the AVP-induced Rho inactivation and the AVP-induced F-actin depolymerization. The data suggest that the signaling pathway underlying the diuretic effects of PGE_2 and probably those of other diuretic agents include cAMP- and Ca^{2+}-independent Rho activation and F-actin formation.

Key words: PGE_2, Aquaporin, AQP2, Rho, Vasopressin, Kidney

Introduction

Arginine-vasopressin (AVP) induces the translocation of water channels (aquaporin-2, AQP2) from intracellular vesicles mainly into the apical membranes of renal collecting duct principal cells [shuttle hypothesis (for reviews, see Wade et al., 1981; Klussmann et al., 2000; Nielsen et al., 2002)]. The AQP2 shuttle constitutes the molecular basis of AVP-regulated antidiuresis. It is initiated by binding of AVP to vasopressin V2 receptors (V2 receptors), mainly located on the basolateral surface of principal cells. The agonist-occupied V2 receptor activates adenylyl cyclase via G_s (Birnbaumer et al., 1992). The resulting increase in cAMP leads to activation of protein kinase A (PKA), phosphorylating, among other substrates, AQP2. The phosphorylation by PKA and also the tethering of PKA by protein kinase A anchoring proteins (AKAP) to unknown compartments are prerequisites for the shuttle (Klussmann et al., 1999; Klussmann et al., 2000; Klussmann et al., 2001a; Katsura et al., 1997; Nielsen et al., 2002). Another prerequisite for the AVP-induced translocation of AQP2 appears to be the inactivation of the small GTP-binding protein Rho and the depolymerization of F-actin (Klussmann et al., 2001b; Valenti et al., 2000; Tamma et al., 2001).

Prostaglandins (PGs) act as autocrine and paracrine lipid mediators, controlling many physiological processes (Funk, 2001; Breyer and Breyer, 2000). The effects of prostaglandin E_2 (PGE_2) on the osmotic water permeability of the collecting duct have been investigated by using various animal models. In the absence of AVP, basolaterally or luminally administered PGE_2 increases basal osmotic water permeability in rabbit cortical collecting ducts, most likely by stimulation of cAMP synthesis via G_s/adenylyl cylase (Sakairi et al., 1995). In contrast, in AVP-stimulated rabbit cortical collecting ducts, basolaterally administered PGE_2 inhibits osmotic water permeability. The effect has been ascribed to inhibition of cAMP synthesis via G_i/adenylyl cyclase (Sonnenburg et al., 1988). In addition, PGE_2 induces elevation of cytosolic Ca^{2+} in rabbit collecting ducts by stimulating both a release from intracellular stores and influx from the extracellular medium. Elevation of cytosolic Ca^{2+}, via the G protein G_q, has also been suggested to contribute to the diuretic effect of PGE_2 (Hebert et al., 1990; Hebert et al., 1993; Hebert, 1994). In the rat terminal inner medullary collecting duct, PGE_2 alone has no effect on basal osmotic water permeability, but attenuates AVP-induced increases in osmotic water permeability. Again, this was suggested to be due to elevation of cytosolic Ca^{2+} (Nadler et al., 1992).

PGE_2 interacts with four different G protein-coupled receptors designated EP_1, EP_2, EP_3 and EP_4 (Coleman et al., 1994; Narumiya et al., 1999; Breyer and Breyer, 2001; Namba et al., 1993; Hatae et al., 2002). The EP receptor subtypes expressed by principal cells have not been identified, but the inhibitory effect of PGE_2 on the AVP-induced increase in osmotic water permeability is likely to be mediated by EP_1

and/or EP_3 receptors, which are coupled to the G_q/phospholipase C (PLC) and G_i/adenylyl cyclase system, respectively (Sakairi et al., 1995). This assumption is supported by the finding that the stable PGE_2 analogue, sulprostone, a selective EP_1/EP_3 receptor agonist, inhibits the AVP-induced increases in osmotic water permeability in the rabbit cortical collecting duct (Hebert et al., 1993; Hebert, 1994). However, the underlying signal transduction pathways are not understood. EP_3 receptors, in addition to coupling to the G_i/adenylyl cyclase system, mediate Rho activation, most likely through the G proteins $G_{12/13}$, and subsequently the formation of stress fibers (Negishi et al., 1995; Hasegawa et al., 1997; Aoki et al., 1999). Activated Rho poses a block to the AVP-induced AQP2 shuttle in principal cells (Klussmann et al., 2001b; Tamma et al., 2001). Thus, we hypothesized that PGE_2 exerts its diuretic effect through activation of Rho *via* EP_3 receptors (see above). To investigate this possibility, the effects of sulprostone, combined with an EP_1 receptor antagonist, SC19220, on the cellular localization of AQP2, on Rho activity and on F-actin were analyzed using primary cultured rat inner medullary collecting duct (IMCD) cells. In addition, levels of cytosolic Ca^{2+} and adenylyl cyclase activity were determined. Our data suggest that activation of the $G_{12/13}$/Rho pathway rather than inhibition of adenylyl cyclase or stimulation of PLC (increase in cytosolic Ca^{2+}) underlies the EP_3 receptor-mediated diuretic action of PGE_2 observed in the presence of AVP. In addition, they provide strong evidence for a central role of Rho in both diuretic and antidiuretic responses.

Materials and Methods

Materials

AVP was synthesized by Dr M. Beyermann, Forschungsinstitut für Molekulare Pharmakologie, Berlin. Sulprostone and SC19220 were purchased from Cayman Chemical, Alexis Biochemicals, Grünberg, Germany, and carbachol, PGE_2, forskolin, dibuyryl cAMP (Bt_2cAMP) and thapsigargin from Sigma, Deisenhofen, Germany. [^{32}P]cAMP and myo-[2-^{3}H]inositol were purchased from Amersham Pharmacia Biotech, Freiburg, Germany. *Clostridium difficile* toxin B was kindly provided by Dr K. Aktories, Albert-Ludwigs-Universität, Freiburg, Germany. The plasmid encoding a fusion protein consisting of glutathion S-transferase (GST) and the Rho-binding domain of the Rho effector Rhotekin was kindly provided by Dr M. A. Schwartz (The Scripps Research Institute, La Jolla, CA, USA).

Culture of IMCD cells, immunofluorescence microscopy and quantification of immunofluorescence intensities

IMCD cells were obtained from rat renal inner medullae and cultured on cover slips as described previously (Maric et al., 1998). Bt_2cAMP was added to the culture medium for maintenance of AQP2 expression. Bt_2cAMP was removed 16 hours prior to experiments, which were performed 6 days after seeding. AQP2 was detected by confocal laser scanning microscopy (LSM 410; Carl Zeiss, Jena, Germany) using specific antibodies raised against the C terminus of AQP2 and Cy3-conjugated anti-rabbit secondary antibodies (Klussmann et al., 1999; Klussmann et al., 2001b; Maric et al., 1998). F-actin was detected after staining with tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated phalloidin by confocal laser scanning microscopy [LSM 410 (Klussmann et al., 2001b)].

For quantification of the effects of AVP, forskolin, Bt_2cAMP, sulprostone and SC19220 on AQP2 localization, the ratio of intracellular/plasma membrane fluorescence intensities was calculated as described previously (Klussmann et al., 1999; Klussmann et al., 2001b). For all groups, mean and standard error values were calculated. Statistical analyses were performed using the Student's *t*-test and one-way analysis of variance (Klussmann et al., 1999; Klussmann et al., 2001b).

Rho pull-down assay and western blotting

The pull-down of active GTP-bound Rho from IMCD cells was essentially carried out as described previously (Ren and Schwartz, 2000). In brief, IMCD cells were grown in 60 mm dishes and incubated with agonists as indicated. GTP-Rho was precipitated from lysates derived from 7 confluent dishes of IMCD cells using the GST-Rhotekin fusion protein (20-30 μg) coupled to glutathion Sepharose 4B. GTP-Rho was eluted by boiling the precipitate in Laemmli buffer (10 minutes) containing DTT (40 mM). Total RhoA in IMCD cell lysates and precipitated GTP-RhoA were detected by western blot analysis using commercially available anti-RhoA monoclonal antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) and peroxidase-conjugated anti-mouse secondary antibodies. Signals were visualized using the Lumi-light western blot detection system and a Lumi-Imager F1 (Roche Diagnostics, Mannheim, Germany). To quantify the amount of active RhoA, signal densities were determined and related to the signal densities obtained for total RhoA. Ratios obtained for the various experimental conditions were normalized to those ratios obtained for control cells. Statistical analysis was carried out using the Newman-Keuls multiple comparison test.

Adenylyl cyclase assay

Preparation of crude membrane fractions and the adenylyl cyclase assay were carried out as described previously (Oksche et al., 1996; Schülein et al., 1996). [^{32}P]cAMP was isolated using the two column method (Salomon et al., 1974). Statistical analysis was carried out using the unpaired *t*-test for independent single assay results.

Inositol-1,4,5-trisphosphate (InsP_3) assay

IMCD cells were grown in 24-well plates. Five days after seeding, the culture medium was replaced by Bt_2cAMP-free medium containing 74 kBq/ml myo-[2-^{3}H]inositol (specific activity 37 MBq/ml). For the uptake of myo-[2-^{3}H]inositol, the cells were grown for 20 hours at 37°C. The cells were washed with Bt_2cAMP-free culture medium containing 10 mM LiCl and further incubated in Bt_2cAMP-free medium containing agonists as indicated. Total inositolphosphates were assayed as described previously (Kirk et al., 1990). Changes in the content of total inositolphosphates are largely due to the formation of inositol-1,4,5-trisphosphate (InsP_3) and therefore described as changes in InsP_3 content.

Determination of cytosolic Ca^{2+}

Measurements of cytosolic Ca^{2+} were essentially performed as described previously (Schaefer et al., 2000). In brief, IMCD cells were grown to confluency on glass coverslips. Fura-2-AM (Molecular Probes, Leiden, The Netherlands) loading was carried out by incubation of the cells in Hepes-buffered saline (128 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM glucose, 0.5% bovine serum albumine, 10 mM Hepes, pH 7.4, 10 μM fura-2-AM; 30 minutes, 37°C). Coverslips were mounted in a custom-made chamber, overlaid with HBS buffer and placed on an inverted epifluorescence microscope (Axiovert 100, Carl Zeiss, Jena, Germany) equipped with a monochromator (Polychrome II, TILL-Photonics, Martinsried, Germany). Agonists were added as indicated. Thapsigargin was applied at the end of each experiment to prevent the internal Ca^{2+} stores limiting the responses to sequentially applied agonists. Fura-2 was alternatively excited at 340, 358, and 380 nm. Emitted light was filtered through a 505-nm long-pass filter and recorded with a 12 bit

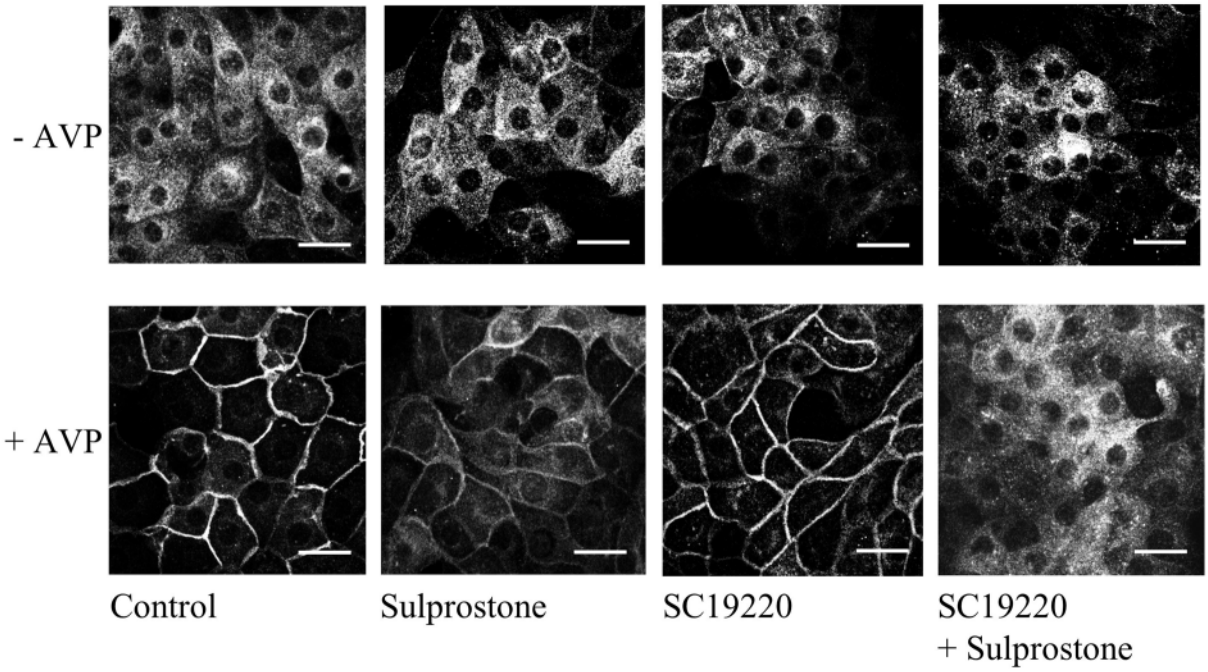

Fig. 1. The effect of EP_3 receptor stimulation on the localization of AQP2 in IMCD cells. IMCD cells were left untreated (control) or incubated with AVP (100 nM; 15 minutes), the EP_1/EP_3 receptor agonist sulprostone (1 μM; 30 minutes), or the EP_1 receptor antagonist SC19220 (10 μM; 40 minutes). If cells were incubated with both sulprostone and SC19220, the SC19220 (10 μM) was added 10 minutes prior to sulprostone (1 μM). AVP was present during the final 15 minutes. After completion of incubations, cells were fixed, permeabilized, and incubated with rabbit anti-AQP2 antibody and secondary Cy3-conjugated anti-rabbit antibodies. AQP2 immunofluorescence was detected by laser scanning microscopy. Scale bars : 20 μm.

cooled CCD camera (Imago, TILL-Photonics). Cytosolic Ca^{2+} concentrations were calculated as described (Grynkiewicz et al., 1985). In each imaging experiment, data from 30-80 individual cells were collected.

Results

Stimulation of EP_3 receptors inhibits AVP-induced AQP2 translocation in IMCD cells

Primary cultured rat IMCD cells were used to determine the effect of EP_3 receptor stimulation on the cellular localization of AQP2. This model system preserves key features of principal cells in situ. In particular, IMCD cells contain the molecular machinery required for the AVP-induced AQP2 translocation. However, for unknown reasons AQP2 predominantly inserts into the basolateral plasma membranes of IMCD cells in response to AVP (Klussmann et al., 1999; Klussmann et al., 2001b; Maric et al., 1998; Maric et al., 2001; Lorenz et al., 2003). Fig. 1 (laser scanning microscopy) and Fig. 2 [quantitative and statistical analysis (Klussmann et al., 1999; (Klussmann et al., 2001b)] show a mainly intracellular distribution of AQP2 under control conditions. AVP induced the translocation of AQP2 to the plasma membrane. The EP_1/EP_3 receptor agonist sulprostone, the EP_1 receptor antagonist SC19220 or a combination of both did not modify the intracellular localization of AQP2 in non-stimulated IMCD cells. Likewise, incubation of the cells with SC19220 alone had no effect on the AVP-induced translocation of AQP2. However,

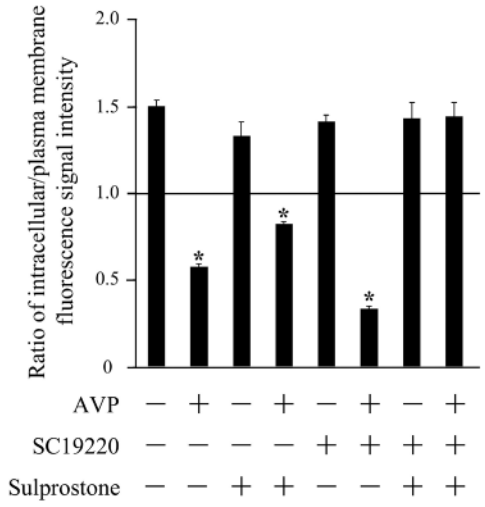

Fig. 2. Quantitative analysis of the effect of EP_3 receptor stimulation on the localization of AQP2 in IMCD cells. IMCD cells were treated as indicated in the legend to Fig. 1. AQP2 immunofluorescence signals were detected by laser scanning microscopy. The intracellular and plasma membrane fluorescence signal intensities were determined and related to nuclear signal intensities. Ratios of intracellular/plasma membrane signal intensities were calculated ($n \geq 20$ cells for each condition tested; mean ± s.e.; three independent experiments). Ratios >1 indicate a mainly intracellular distribution of AQP2, and ratios <1 indicate a predominant localization at the plasma membrane. *, values significantly different from untreated control cells ($P<0.001$).

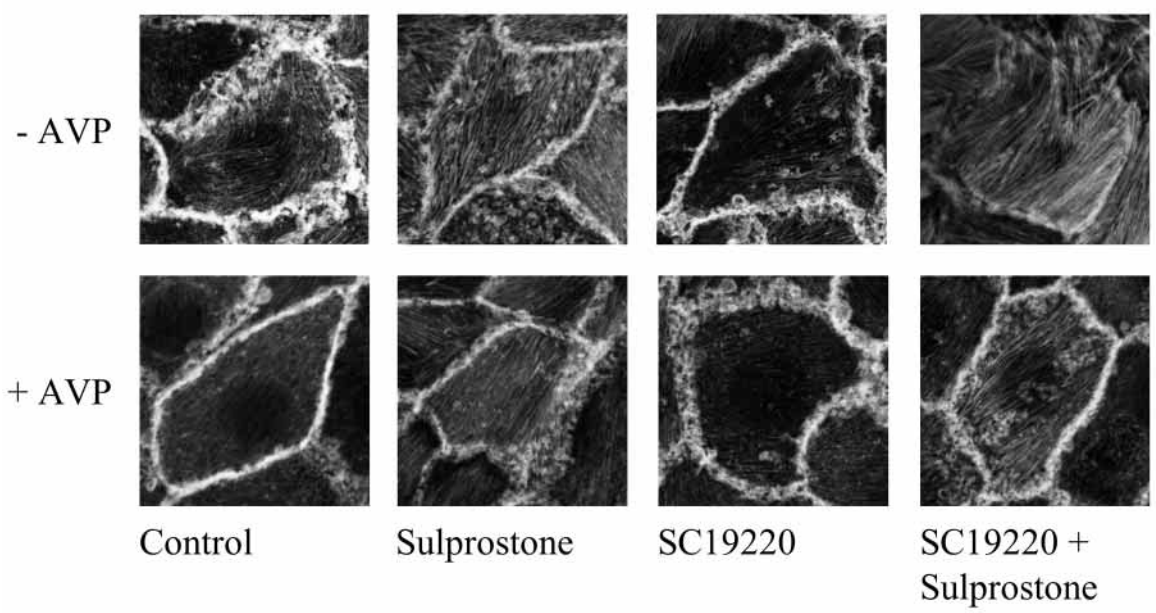

Fig. 3. The effect of EP_3 receptor stimulation on the F-actin cytoskeleton in IMCD cells. IMCD cells were incubated with the indicated agents as described in the legend to Fig. 1. After completion of the incubations, cells were fixed, permeabilized and incubated with TRITC-conjugated phalloidin (0.1 mg/ml). Phalloidin fluorescence was detected by laser scanning microscopy. Shown are single representative cells from at least three independent experiments for each condition.

sulprostone alone or combined with SC19220 reduced or abolished, respectively, the AVP-induced AQP2 translocation. The results indicate that stimulation of EP_3 receptors abolishes AVP-induced AQP2 translocation in IMCD cells. This inhibitory effect appears to be attenuated by EP_1 receptor stimulation.

Stimulation of EP_3 receptors induces the formation of stress fibers in IMCD cells

EP_3 receptor-mediated activation of Rho and subsequent formation of stress fibers has recently been reported (Hasegawa et al., 1997). In IMCD cells, activation of Rho is accompanied by formation of stress fibers and prevents AQP2 translocation in response to elevation of cAMP (Klussmann et al., 2001b). Therefore, the ability of sulprostone to induce the formation of stress fibers in IMCD cells was examined. Stress fibers were detected by staining F-actin with TRITC-conjugated phalloidin and visualization by laser scanning microscopy (Fig. 3). As reported, AVP caused a decrease of stress fibers (Klussmann et al., 2001b). In contrast, sulprostone alone or combined with SC19220 induced the formation of stress fibers under both conditions, i.e. in the presence or absence of AVP. SC19220 alone did not influence the content of stress fibers in non-stimulated cells, nor did it inhibit the AVP-induced depolymerization of stress fibers. The data suggest that stimulation of the V2 and EP_3 receptor by AVP and sulprostone/SC19220, respectively, has opposing effects on the F-actin cytoskeleton in IMCD cells.

Bidirectional control of Rho by antidiuretic and diuretic agents in IMCD cells

We have previously shown that AVP and *C. difficile* toxin B induce a depolymerization of F-actin in IMCD cells (Klussmann et al., 2001b) (Fig. 3). Here, we determined the effects of AVP, toxin B and of sulprostone on RhoA activity (Fig. 4A,C). Active (GTP-bound) RhoA was quantitatively analyzed by pull-down assays, using the Rho-binding domain of Rhotekin fused to GST (Ren and Schwartz, 2000). AVP and toxin B caused a decrease in the amount of active RhoA compared to control cells (Fig. 4A,C). In contrast, sulprostone, alone or combined with SC19220, increased RhoA activity (Fig. 4B,C). AVP, added to cells preincubated with sulprostone alone, decreased RhoA activity but the level of RhoA activity observed in cells incubated with AVP alone was not reached (Fig. 4C). In cells incubated with sulprostone, SC19220 and AVP, RhoA activity was similar to that of control cells, indicating that selective EP_3 receptor stimulation abolished the AVP-mediated inhibition of RhoA (Fig. 4B,C). The data reveal a bidirectional control of RhoA activity in IMCD cells with the antidiuretic agent AVP inhibiting and the diuretic agent sulprostone (combined with SC19220) stimulating it. In addition, the data suggest that in the presence of AVP the EP_3 receptor-induced stimulation of RhoA is attenuated by EP_1 receptor activation (see above; Figs 1 and 2).

EP_3 receptor stimulation prevents AQP2 translocation independently of cAMP

Our results indicate that Rho activation is the cellular mechanism underlying EP_3 receptor-mediated diuresis. However, previous studies suggested that inhibition of the G_i/adenylyl cyclase system contributes to the diuretic effect of PGE_2 (Sonnenburg et al., 1988; Breyer and Breyer, 2001). Therefore, the effect of sulprostone on AVP-stimulated adenylyl cyclase activity was determined in IMCD cell membrane preparations (Fig. 5). Sulprostone did not stimulate

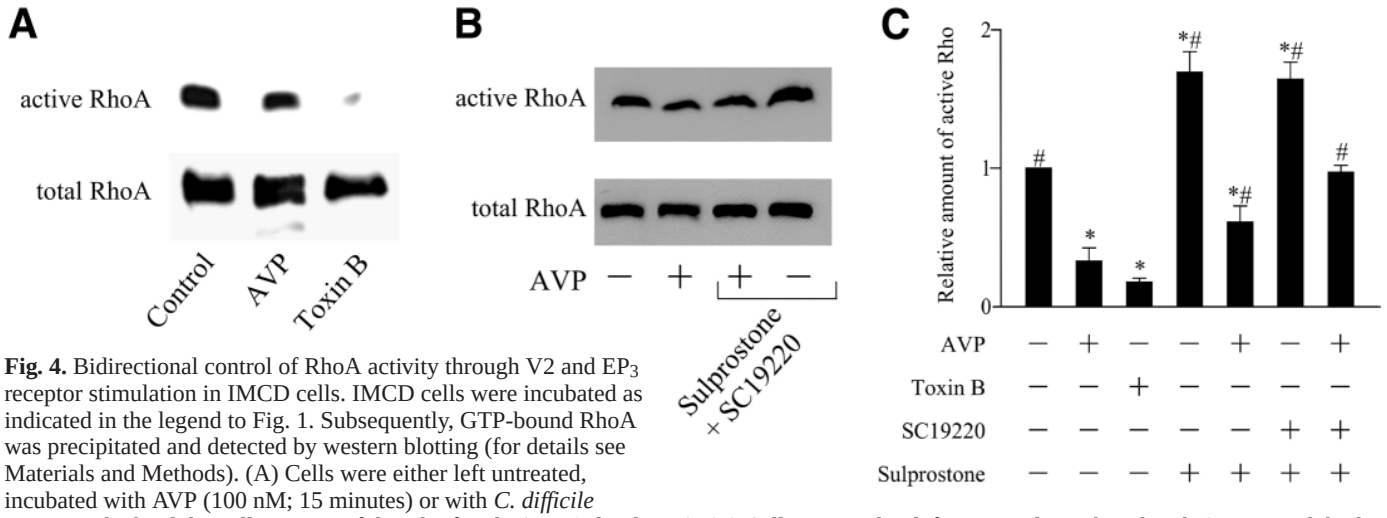

Fig. 4. Bidirectional control of RhoA activity through V2 and EP_3 receptor stimulation in IMCD cells. IMCD cells were incubated as indicated in the legend to Fig. 1. Subsequently, GTP-bound RhoA was precipitated and detected by western blotting (for details see Materials and Methods). (A) Cells were either left untreated, incubated with AVP (100 nM; 15 minutes) or with *C. difficile* toxin B, which inhibits all proteins of the Rho family (4 μg/ml; 4 hours). (B) Cells were either left untreated, incubated with AVP or with both sulprostone and SC19220 in the absence or presence of AVP. (C) For quantification, immunoblots were densitometrically analyzed (for details see Materials and Methods). RhoA activity is indicated by the amount of GTP-bound RhoA related to the amount of total RhoA in the whole cell lysates. Values represent amounts of RhoA normalized to untreated control cells. Results are means ± s.e. of at least three independent experiments per condition (n=3-6). *, statistically different from untreated control cells (for untreated control cells versus sulprostone-treated cells P<0.01, for all other comparisons P<0.001); #, statistically different from AVP-stimulated cells (for AVP-stimulated cells versus + AVP + sulprostone P<0.05, for all other comparisons P<0.001).

adenylyl cyclase activity but reduced the AVP-stimulated adenylyl cyclase activity by about 18%.

To test whether EP_3 receptor-mediated inhibition of adenylyl cyclase is relevant for the inhibition of AVP-induced AQP2 translocation, the effects of sulprostone and SC19220 on the AQP2 shuttle were determined in IMCD cells exposed to either high levels of dibutyryl cyclic adenosine monophosphate (Bt_2cAMP, 500 μM) or forskolin (100 μM), a strong, direct activator of adenylyl cyclase. Fig. 6 shows that even under these conditions, sulprostone alone or combined with SC19220, maintained its ability to inhibit the AQP2 shuttle, indicating that the inhibition of the AQP2 shuttle through EP_3 receptors is independent of cellular cAMP levels. Thus, as in many other systems, the receptor-mediated inhibition of adenylyl cyclase does apparently not contribute to the cellular response. A quantitative analysis of the cellular distribution of AQP2 is shown in Fig. 7 [compare Fig. 2 (Klussmann et al., 1999; Klussmann et al., 2001b)].

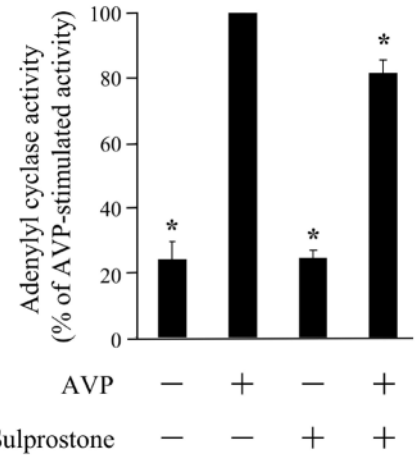

Fig. 5. Determination of adenylyl cyclase activity in membrane preparations of IMCD cells. Crude membrane fractions of IMCD cells were prepared, and adenylyl cyclase activity was determined (30 μg protein/reaction). Membranes were left untreated, incubated with AVP (100 nM; 15 minutes), sulprostone (1 μM; 30 minutes) or sulprostone (1 μM; 30 minutes) with AVP (100 nM) added for the final 15 minutes. Data represent the mean ± s.e. of three independent experiments and are calculated as the percentage of AVP-stimulated activity. Absolute values for adenylyl cyclase activity (pmol/mg protein/minute) in the three independent experiments were: basal 15.1, 11.3 and 6.82; AVP: 76.7, 35.7 and 32.8; sulprostone: 21.0, 7.7 and 7.9; sulprostone and AVP: 66.5, 28.8 and 25.3. *, values significantly different from AVP-stimulated control (P<0.05).

EP_3 receptor stimulation neither induces formation of InsP_3 nor elevation of cytosolic Ca^{2+} in IMCD cells

Elevation of cytosolic Ca^{2+} in response to PGE_2/sulprostone stimulation of rabbit cortical collecting ducts has also been suggested to contribute to the inhibitory effect of PGE_2 on AVP-induced increases in osmotic water permeability (Hebert, 1994). We, therefore, investigated the effects of sulprostone on the formation of InsP_3 and cytosolic Ca^{2+} levels in IMCD cells. Fig. 8A shows that AVP and the muscarinic receptor/G_q-stimulating agonist carbachol induced statistically significant 1.4- and 2.4-fold increases in InsP_3 respectively; in contrast, sulprostone failed to induce InsP_3 formation in IMCD cells. Cytosolic Ca^{2+} was imaged in single, fura-2-loaded IMCD cells (data not shown). In agreement with other reports (Nasrallah et al., 2001; Lorenz et al., 2003), AVP, sulprostone and PGE_2 induced a small rise in cytosolic Ca^{2+} (from about 50 nM to less than 200 nM) in a small number of IMCD cells tested (2.4, 1.4 and 1.4%, respectively; Fig. 8B). Preincubation of cells with SC19220 invariably abolished the elevation of cytosolic Ca^{2+} in

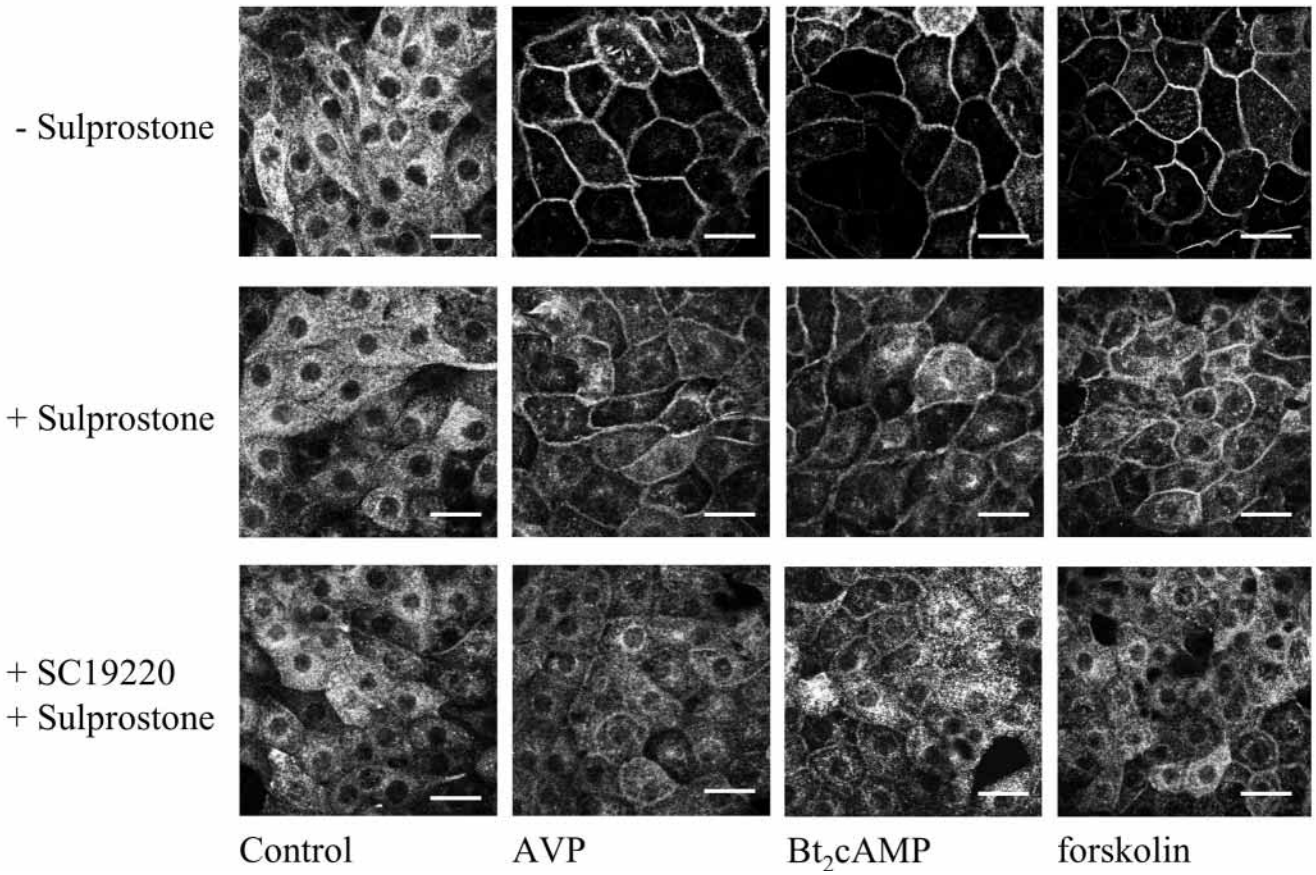

Fig. 6. The effect of EP_3 receptor stimulation on the localization of AQP2 in IMCD cells in the presence of high levels of cAMP. IMCD cells were left untreated (control) or incubated with AVP (100 nM; 15 minutes), Bt_2cAMP (500 μM; 15 minutes), forskolin (100 μM; 15 minutes), a strong, direct activator of adenylyl cyclase, and with or without sulprostone (1 μM; 30 minutes). If cells were incubated with both sulprostone and SC19220, SC19220 (10 μM) was added 10 minutes prior to sulprostone. AVP, Bt_2cAMP or forskolin were present during the final 15 minutes. After completion of the incubations, cells were fixed, permeabilized, and incubated with rabbit anti-AQP2 antibody and secondary Cy3-conjugated anti-rabbit antibodies. AQP2 immunofluorescence was detected by laser scanning microscopy. Scale bars: 20 μm.

response to sulprostone or PGE_2, indicating that the cytosolic Ca^{2+} signals resulted from EP_1 receptor stimulation (see Introduction). The data suggest that EP_3 receptor stimulation does not induce the formation of Ins*P*$_3$ or an increase in cytosolic Ca^{2+} in IMCD cells. Thus, Ca^{2+} is not involved in EP_3 receptor-mediated stimulation of Rho and inhibition of the AVP-induced AQP2 shuttle.

Discussion

AVP regulates antidiuresis by inducing the translocation of AQP2 into the apical plasma membranes of collecting duct principal cells. This step is prevented by activation of RhoA (Klussmann et al., 2001b; Tamma et al., 2001). The action of AVP is antagonized by several diuretic agents including endothelin-1, bradykinin and PGE_2. The molecular mechanism is not known. We show that the synthetic PGE_2 analogue sulprostone, through EP_3 receptors, stimulates RhoA in IMCD cells in a cAMP- and Ca^{2+}-independent manner and antagonizes both the AVP-induced (V2 receptor-mediated) inhibition of RhoA and the AVP-induced AQP2 translocation. These findings point to a central role of Rho in the diuretic response. The presence of EP_3 receptors in the human collecting duct (Breyer and Breyer, 2000; Breyer et al., 1996a; Breyer et al., 1996b; Morath et al., 1999) suggests that the mechanism we characterized is also operable in the human kidney. A model of the proposed mechanism is depicted in Fig. 9.

The EP_3 receptor-induced activation of RhoA in IMCD cells is, most likely, mediated by the G proteins $G_{12/13}$ (Namba et al., 1993; Hasegawa et al.,1997; Katoh et al., 1996; Nakamura et al., 1998; Yamaguchi et al., 2000; Hatae et al., 2002). $G_{12/13}$ directly activate Rho guanine nucleotide exchange factors (RhoGEFs, e.g. p115) which in turn activate RhoA (Wells et al., 2002). Neither cAMP nor Ca^{2+} are involved in this pathway. In contrast, the inhibition of RhoA through V2 receptors most likely involves cAMP. AVP stimulates cAMP synthesis and subsequent activation of PKA which may phosphorylate Rho (Lang et al., 1996; Forget et al., 2002). We have recently shown that the forskolin-induced AQP2 shuttle in CD8 cells (see Introduction) is accompanied by RhoA phosphorylation, a decrease in RhoA activity, and an increased interaction of RhoA with Rho guanine nucleotide dissociation inhibitor [RhoGDI (Tamma et al., 2003)], the protein that terminats Rho activity (Forget et al., 2002). Accordingly, activation of RhoA *via* $G_{12/13}$ following the stimulation of EP_3 receptors and inhibition of RhoA by cAMP-dependent phosphorylation, following the stimulation of V2 receptors, are most likely the pathways for the bidirectional control of RhoA in IMCD cells.

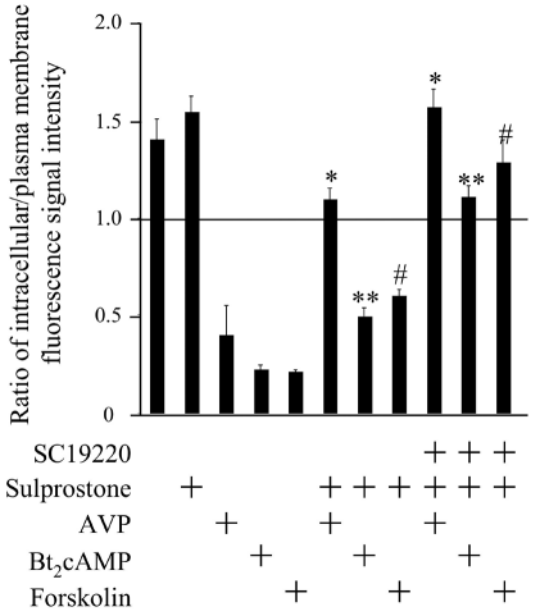

Fig. 7. Quantitative analysis of the effect of EP_3 receptor stimulation on the localization of AQP2 in IMCD cells in the presence of high levels of cAMP. IMCD cells were treated as indicated in Fig. 6. AQP2 immunofluorescence signals were detected by laser scanning microscopy and the ratios of intracellular/plasma membrane signal intensities were determined as described in the legend to Fig. 2 (*n*=20 cells for each condition tested; mean ± s.e.; three independent experiments). *, ** and #, values significantly different from AVP-, Bt_2cAMP- and forskolin-stimulated cells respectively (P<0.001).

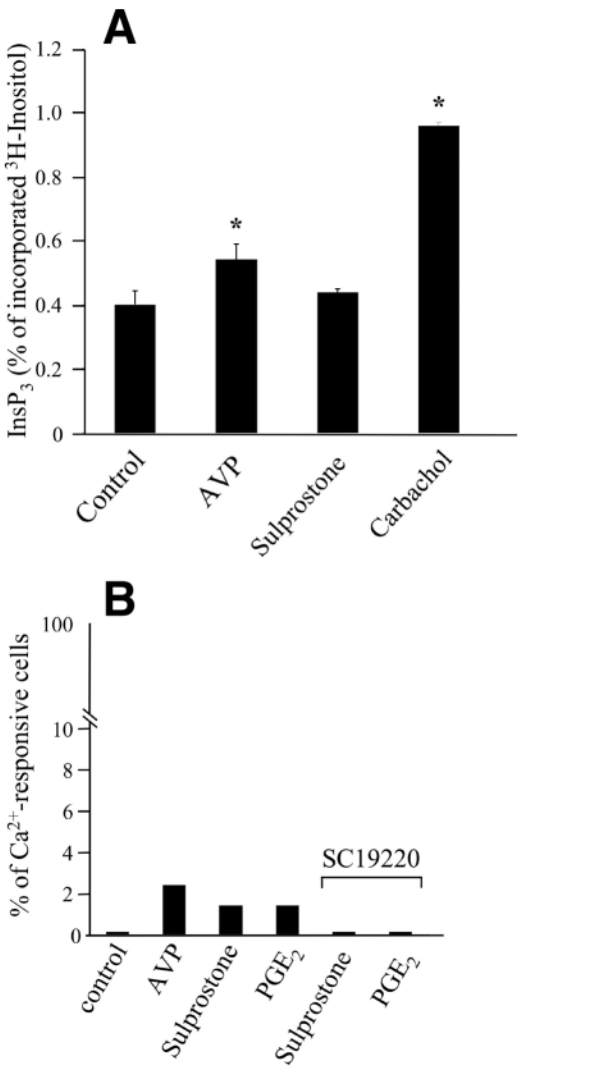

Fig. 8. Inositol-1,4,5-trisphosphate (InsP_3) formation in and Ca^{2+}-responsiveness of IMCD cells. (A) IMCD cells were incubated with myo-[2-^{3}H]inositol for 20 hours at 37°C in culture medium without Bt_2cAMP. Thereafter, cells were incubated with AVP (100 nM), sulprostone (1 μM) or carbachol (100 μM; 60 minutes for each agonist). Subsequently, radioactive InsP_3 was isolated as described in Materials and Methods. One out of three independent experiments yielding similar results is shown. InsP_3 is expressed as a percentage of total [2-^{3}H]inositol incorporated by IMCD cells. *, statistically different from untreated control cells (P<0.05). (B) Cytosolic Ca^{2+} was determined in IMCD cells (for details see Materials and Methods) and the percentage of cells responding to stimulation with AVP (100 nM), sulprostone (1 μM) or PGE_2 (1 μM) by elevation of cytosolic Ca^{2+} was determined. Control cells were left untreated. If indicated, the EP_1 receptor antagonist SC19220 (10 μM) was added 10 minutes prior to the experiment. The cytosolic Ca^{2+} concentration in untreated cells was about 50 nM; stimulation by AVP, sulprostone or PGE_2 induced an elevation to 200 nM in the responding cells.

The mechanism by which activated Rho and its effectors inhibit AVP-induced AQP2 translocation apparently involves the F-actin cytoskeleton. F-actin, in particular subapical F-actin, also referred to as the terminal web, has a barrier function in many exocytic processes. Its disintegration is considered a prerequisite for exocytosis in various cell types including chromaffin cells, mast cells and pancreatic acinar cells (Valentijn et al., 1999). Similarly, the F-actin network (stress fibers) in IMCD cells may function as a physical barrier which hinders AQP2-bearing vesicles reaching the plasma membrane. Several lines of evidence support this view. Activation of RhoA *via* EP_3 receptors or expression of constitutively active RhoA lead to the formation of stress fibers and inhibited AVP-induced AQP2 translocation in IMCD cells [see above and Klussmann et al. (Klussmann et al., 2001b)]. Effectors of activated Rho that promote the formation of stress fibers are the Rho kinases (Tapon and Hall, 1997). Inhibition of Rho kinases with the inhibitor Y-27632 reduces the content of stress fibers in IMCD and CD8 cells and induces translocation of AQP2 independently of cAMP elevation (Klussmann et al., 2001b; Tamma et al., 2001). Similarly, depolymerization of stress fibers induced by cytochalasin D allows AQP2 translocation without elevation of cAMP in IMCD and CD8 cells (Klussmann et al., 2001b; Tamma et al., 2001). In addition to its barrier function, F-actin may directly interact with AQP2 (Brown et al., 1998; Umenishi et al., 2000).

The inhibitory effect of PGE_2 *via* EP_3 receptors on AVP-induced increases in osmotic water permeability has been ascribed to an inhibition of adenylyl cyclase activity (Hebert, 1994; Breyer and Breyer, 2001). However, pertussis toxin, an inhibitor of G_i, does not prevent PGE_2 antagonizing the effects of AVP in cultured rabbit cortical collecting duct cells or in isolated rat outer medullary collecting ducts (Noland et al., 1992; Aarab et al., 1993; Aarab et al., 1999). In IMCD and

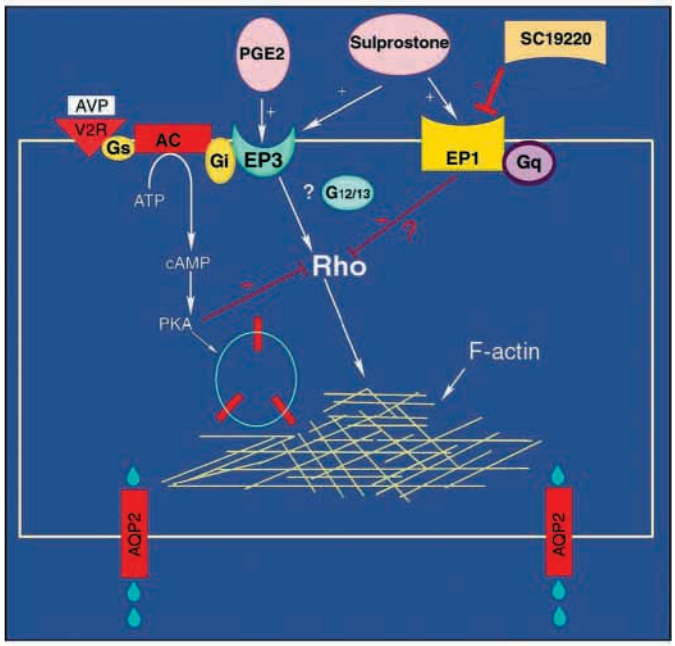

Fig. 9. A model for the involvement of Rho in prostaglandin E_2 (PGE_2)-antagonized vasopressin-mediated water reabsorption. Vasopressin (AVP) facilitates water reabsorption in renal collecting duct principal cells by binding to vasopressin V2 receptors (V2R). The agonist-occupied V2R activates adenylyl cyclase (AC) *via* the G protein G_s. The resulting increase in cAMP leads to activation of protein kinase A (PKA), phosphorylating AQP2 and Rho. Rho phosphorylation decreases its activity, resulting in the depolymerisation of F-actin and facilitating the insertion of AQP2 predominantly into the apical plasma membrane. Stimulation of EP_3 receptors by PGE_2, achieved by incubation of IMCD cells with a combination of the PGE_2 analogue sulprostone, an EP_1/EP_3 receptor agonist and the EP_1 receptor antagonist SC19220, induces the activation of Rho, most probably via the G proteins $G_{12/13}$. Rho activation is independent of increases in cAMP and cytosolic Ca^{2+}. It promotes the formation F-actin which hinders AQP2-bearing vesicles reaching the plasma membrane by acting as a physical barrier. EP_3 receptor-mediated activation of the G protein G_i, inhibiting adenylyl cyclase, is unlikely to contribute to the diuretic effect of PGE_2. In the presence of AVP, the EP_3 receptor-induced Rho activation and inhibition of AQP2 translocation are attenuated by EP_1 receptor stimulation; the underlying signaling pathway is not known.

CD8 cells, pertussis toxin inhibits the AQP2 shuttle, presumably by inhibition of G_{i3} located on AQP2-bearing vesicles (Valenti et al., 1998). Therefore, it cannot be used to test the involvement of adenylyl cyclase in the inhibitory effect of sulprostone on the AQP2 shuttle. However, we show here that EP_3 receptor activation leads to inhibition of the AQP2 shuttle despite high levels of cAMP (Figs 6 and 7), strongly suggesting that inhibition of adenylyl cyclase does not contribute to the diuretic effect.

Endothelin-1 and bradykinin antagonize AVP-induced antidiuresis by stimulation of their cognate receptors (ET_B- and B_2 receptors respectively) located on principal cells (for a review, see Klussmann et al., 2000). ET_B receptors couple to both the G_q/PLC system and G_i/adenylyl cyclase; B_2 receptors activate the G_q/PLC system. Therefore, inhibition of cAMP synthesis and elevation of cytosolic Ca^{2+} have been suggested to contribute to the diuretic effects of these agents. However, both receptors also mediate Rho activation (Gohla et al., 1999; Kitamura et al., 1999). In analogy to the EP_3 receptor signaling, we propose that diuretic receptors like ET_B and B_2 receptors exert their diuretic effects by activating the $G_{12/13}$/Rho pathway.

The identification of Rho and its effectors, the Rho kinases, as central regulators of water reabsorption opens the door to new therapeutic concepts for the treatment of diseases characterized by disturbed water homeostasis, e.g. nephrogenic diabetes insipidus (NDI) or other diuretic states. Congenital NDI is mainly caused by mutations in the V2 receptor (Oksche and Rosenthal, 1998). The inactivation of Rho or Rho kinases may induce the translocation of AQP2 independently of functional V2 receptors and thus reduce the loss of water. Inhibitors of Rho kinases (Y-27632 and hydroxyfasudil) are currently being tested or approved for the treatment of several diseases (Wettschurek and Offermanns, 2002). The use of these substances is limited owing to the fact that they cannot be applied in a tissue-specific manner. An alternative approach is the retrograde transfer of genes into the tubular system of the kidney (Moullier et al., 1994). Candidate genes are dominant negative mutants of Rho or Rho kinases under control of an inducible version of the collecting duct-specific AQP2 promotor.

Our data suggest that the EP_1 receptor antagonist SC19220 augments the inhibitory effects of sulprostone on AQP2 translocation and Rho activity, implying that activation of the EP_1 receptor reduces the EP_3 receptor-induced diuresis. EP_1 receptors couple to the G_q/PLC system; their activation results in the formation of InsP_3 with subsequent elevation of cytosolic Ca^{2+} and generation of diacylglycerol (DAG) which in turn activates PKC. Recently, it was shown that PKC causes F-actin disassembly through activation of Src kinase, which in turn stimulates the Rho-specific GTPase-activating protein p190, thereby inactivating Rho (Brandt et al., 2002). In addition, PKC phosphorylates G_{12}, thereby attenuating its activity (Fields et al., 1997). However, PKC activation via the PLC pathway is unlikely to account for the EP_1 receptor effect in IMCD cells, since sulprostone does not induce the formation of InsP_3 to a detectable degree and mediates an increase of cytosolic Ca^{2+} in only a minority of cells. It is possible that stimulation of other phospholipases through EP_1 receptors leads to activation of PKC. For example, activation of phospholipase D results in the formation of phosphatidic acid which is converted to DAG in a subsequent step (Newton, 1995; Newton, 1997).

In summary, our data indicate that the signal transduction pathway underlying the diuretic effect of PGE_2 and possibly that of other diuretic agents includes Rho activation without the involvement of a cAMP- or Ca^{2+}-dependent step. In addition, the data suggest that the pharmacological interference with the Rho pathway in principal cells is a strategy suitable for the treating diseases characterized by a disturbed water homeostasis.

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