RhoA exerts a permissive effect on volume-regulated anion channels in vascular endothelial cells

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RhoA exerts a permissive effect on volume-regulated anion channels in vascular endothelial cells. HYPOTONIC CELL SWELLING ACTIVATES an outwardly rectifying anion channels in vascular endothelial cells. An intriguing, but largely unresolved, property of VRACs is their activation mechanism. Under experimental conditions, VRACs are typically activated by cell swelling evoked either by extracellular perfusion with a hypotonic solution or by intracellular dialysis with a hypertonic pipette solution. However, several observations indicate that VRACs can also be activated in the absence of cell swelling, e.g., by reducing intracellular ionic strength (7, 40), by mechanical stimulation (3), or by intracellular application of guanosine 5’-O-(3-thiotriphosphate) (GTPγS) (41). The isovolumic activation has stirred a controversy about the causal relation between cell volume and VRAC activation. Some groups have proposed that VRAC activity is primarily controlled by changes in cell volume but that the cellular volume sensor can be (de)sensitized by other parameters such as intracellular ionic strength (7, 8). Alternatively, it has been proposed that a decrease in intracellular ionic strength (e.g., due to water influx during hypotonic cell swelling) constitutes the critical trigger for channel activation (40). A strong argument in favor of the ionic strength mechanism is that single VRACs could be directly activated by decreased ionic strength (28).

Irrespective of the outcome of this controversy, it has become clear that activation of VRACs during hypotonic cell swelling depends on the specific structural and/or molecular architecture of the cell. For example, hypotonic activation of VRACs is largely deficient in caveolin-1-deficient Caco-2 cells (a colon carcinoma cell line), but transient expression of caveolin-1 in these cells restores the ability of hypotonic cell swelling to activate VRACs. This indicates that VRAC activation during hypotonic cell swelling is at least partly controlled by caveolin-1. In fact, VRACs in caveolin-1-deficient cells exhibit reduced sensitivity to hypotonic cell swelling, and caveolin-1-deficient cells also exhibit reduced sensitivity to VRAC agonists such as guanosine 5’-O-(3-thiotriphosphate) (GTPγS) or C3 exoenzyme. These observations suggest that caveolin-1 plays a role in the regulation of VRAC activity. However, the exact mechanism by which caveolin-1 regulates VRAC activity remains to be elucidated. Further studies are needed to clarify the role of caveolin-1 in the regulation of VRAC activity and to determine the functional significance of this regulation.

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cells recovers VRAC activity (37). Furthermore, efficient activation of VRACs requires, at least in some cell types, a functional F-actin cytoskeleton (19, 31). Finally, the transient activation of VRACs by intracellular application of GTPγS points to a modulatory role for GTP-binding proteins (41). Incubation with Clostridium limosum or C. botulinum C3 toxins that specifically inactivate Rho GTPases (A/B/C isoforms) (15) strongly reduced the activation of VRACs by cell swelling or by intracellular GTPγS (26, 35). Similarly, *C. difficile* toxin B, which is another Rho-inhibiting bacterial toxin, inhibits *I*_{Cl,swell} in N1E115 neuroblastoma cells (10). Taken together, these studies identify Rho GTPases as modulators for VRAC activity.

Rho GTPases (A/B/C isoforms) are GTP-regulated molecular switches that control contractile activity in smooth muscle and the formation of stress fibers in noncontractile cell types (33). The Rho family of GTPases plays important roles in F-actin organization (6). Activation of Rho requires dissociation from a cytosolic binding partner RhoGDI (guanosine nucleotide dissociation inhibitor) and exchange of GDP for GTP, which is catalyzed by Rho guanosine nucleotide exchange factors (RhoGEFs) (6). It has recently been shown that stimulation of some G protein-coupled receptors (e.g., thrombin receptor) increases Rho activity (9, 39). The molecular cascade between G protein-coupled receptor and Rho has also been elucidated: ligand-bound receptors activate heterotrimeric G_{12} and/or G_{13} proteins, of which the dissociated α-subunits (G_{12α} or G_{13α}) stimulate RhoGEF (12, 18, 22).

Once activated, Rho exerts its effects via multiple downstream effectors (6). The Rho-induced stress fiber formation is mediated by mDia1, which promotes actin polymerization and by Rho kinase, a serine/threonine protein kinase that induces myosin light chain phosphorylation by inhibiting myosin light chain phosphatase (6, 33, 42).

We have previously shown that activation of VRACs in calf pulmonary artery endothelial (CPAE) cells (a macrovascular endothelial cell line) requires a functional Rho/Rho kinase/myosin light chain phosphorylation pathway. Indeed, inhibition of Rho, Rho kinase, or myosin light chain kinase precludes swelling-induced activation of VRAC, whereas inhibition of myosin light chain phosphatase exerts a potentiating effect on VRAC (25, 26). In this study, we wanted to establish whether there is a direct and causal link between hypotonicity-induced cell swelling, activation of the Rho pathway, and opening of VRAC. Three implications of this hypothesis were tested: 1) Does activation of the Rho pathway result in an activation of VRAC? 2) Does hypotonic cell swelling activate the Rho pathway? 3) Is a functional Rho pathway a universal requirement for VRAC activation? Our data indicate that cell swelling does not activate the Rho pathway in CPAE cells and, vice versa, that activation of the Rho pathway does not trigger VRAC activation. Furthermore, VRACs are insensitive to GTPγS or Rho inhibition by C3 toxins in caveolin-1-expressing Caco-2 cells. We therefore propose that the Rho pathway exerts a permissive role with respect to VRAC activation in some cell types such as CPAE cells.

**MATERIALS AND METHODS**

**Cells.** We used CPAE (American Type Culture Collection CCL-209) and human colorectal carcinoma cells Caco-2 (ECACC 86010202). The cells were grown in DMEM (Life Technologies, GIBCO) containing 20% FCS, 2 mM l-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin, maintained at 37°C in a fully humidified atmosphere of 10% CO₂ in air. Passage of the cells was performed by brief exposure to 0.5 g/l trypsin in a Ca²⁺- and Mg²⁺-free solution. Only nonconfluent cells were used in the patch-clamp experiments.

Twenty-four hours after the cells were seeded, they were transiently transfected with the bicistronic pcDNA3/IRES-GFP vector (38), containing the cDNA encoding the constitutively active forms of G_{12α} (G_{12α} Asp239Glu), RhoA (RhoA Tyr368Glu), Rho kinase (Rho kinase-CAT), or caveolin-1. CPAE cells were seeded at 2,000–5,000 cells and Caco-2 cells were seeded at 5,000 per 18 mm gelatin-coated coverslip. Transfection was performed using 1 μg DNA, 7 μl Superfect (Reagens Life Technologies), and 2 μl Lipofectamine (Life Technologies) per coverslip. Cells were analyzed on days 2 and 3 after transfection.

**Solutions.** At the beginning of the patch-clamp recordings, a modified Krebs solution containing (in mM) 150 NaCl, 6 KCl, 1 MgCl₂, 10 glucose, and 5 HEPES (pH 7.4) with NaOH was replaced by an isotonic Ca²⁺ solution, containing (in mM) 105 NaCl, 6 CsCl, 1.5 CaCl₂, 10 glucose, 10 HEPES, and 90 mannitol, adjusted to pH 7.4 with NaOH. The osmolality of the solutions, as measured with a vapor pressure osmometer (Wescor 5500, Slag, Gladbach, Germany) was 320 ± 5 mosmol/kgH₂O. The 25% hypotonic solution (HTS) was obtained by omitting 90 mM mannitol from this Ca²⁺ solution. Pipette solutions contained (in mM): 40 CsCl, 100 cesium aspartate, 1 MgCl₂, 1.93 CaCl₂, 5 EGTA, 4 Na₂ATP, and 10 HEPES, adjusted to pH 7.2 with CsOH (290 mosmol/kgH₂O). The concentration of free Ca²⁺ in this solution was buffered at 100 nM.

In some experiments, 100 μM GTPγS (Sigma) was included in the pipette solution. Inactivation of Rho was achieved by incubating cells with the C2IN-C3 fusion toxin as described previously (4, 26). The C2IN-C3 fusion toxin consists of the amino-terminal part of the C2I component of the *C. botulinum* toxin C2 fused to the entire *C. limosum* C3 exoenzyme. Interaction of the membrane-binding protein C2I, responsible for the cellular uptake of C2 with C2IN, allows internalization of the toxin. Cells were incubated overnight with 100–200 ng/ml of C2IN-C3 and 200 ng/ml C2I.

**Electrophysiological recordings.** Transfected green fluorescent cells were visualized in a patch-clamp setup as described previously (38). Currents were monitored with an EPC-7 patch-clamp amplifier (List Electronic, Lambrecht/Pfalz, Germany). Patch electrodes had direct current resistances between 2 and 6 MΩ. An Ag–AgCl wire was used as reference electrode.

Whole cell membrane currents were measured using ruptured patches. Currents were sampled at 1-ms intervals and filtered at 1,000 Hz. The following voltage protocol was applied every 15 s from a holding potential of −25 mV: a step to −80 mV for 0.2 s, followed by a step to −100 mV for 0.1 s, and a 1.5-s linear voltage ramp to +100 mV. A step protocol was also used, consisting of 1-s voltage steps from a holding potential of −25 mV to potentials ranging from −100 to +100 mV.
mV in 20-mV increments applied every 5 s. Experiments were performed at room temperature.

Affinity precipitation of cellular Rho-GTP. BL21 Escherichia coli bacteria were transformed with the pGEX3X vector containing the glutathione S-transferase (GST)-C21 construct (a kind gift of Dr. J. G. Collard) that encodes the Rho-binding domain of Rhotekin (a Rho effector protein) fused to GST. GST-C21 protein synthesis was induced with 0.1 mM isopropyl β-D-thiogalactoside. After 12 h, the culture was centrifuged (3 900 g, 20 min, 4°C), and the pellet was sonicated in a bacterial lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 5% glycerol, 0.1% Triton X-100, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonfyl fluoride (PMSF), 0.4 mM sodium pervanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). The lysate was centrifuged (13 000 g, 20 min, 4°C), and the supernatant containing the GST-C21 fusion protein was incubated with 50% glutathione-Sepharose 4B beads (Amersham) during 60 min at 4°C. The beads were washed three times with and resuspended in GST-fusion buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM EDTA, 5% glycerol, 1% Nonidet P-40, 2 mM DTT, 1 mM PMSF, 0.4 mM sodium pervanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin).

CPAE cells were stimulated with a 25% hypotonic Cs+ solution (see Solutions) or with 5 U/ml thrombin reagent (DADE Behring) in the isotonic Cs+ solution. After different periods of stimulation, cells were lysed in an isotonic or hypotonic 1% Triton buffer (25 mM Tris, 100 mM NaCl, 2 mM DTT, 1 mM EGTA, 1 mM PMSF, 1% Triton X-100, 0.4 mM sodium pervanadate, 10 μg/ml leupeptin, and 90 mM mannitol for the isotonic buffer). Rho-GTP formation was assessed with a pull-down assay (39). Briefly, cell lysates were clarified by centrifugation (1,000 g, 4°C, 10 min) and then incubated at 4°C during constant rotation with GST-C21 (∼50 μg). After 60 min, the beads were precipitated at 500 g for 5 min at 4°C and washed twice with GST-fusion buffer. Bound proteins were released with SDS loading dye, separated on SDS-PAGE, and electroblotted to nitrocellulose membranes. RhoA was detected with a monoclonal antibody (Santa Cruz Biotechnology sc-418; diluted 1:2,000 in Tris-buffered saline (TBS)-T) and a secondary alkaline-phosphatase-conjugated goat anti-mouse IgG (Amersham; diluted 1:7,000). Immunoreactive bands were visualized and quantified using the Vistra enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) on a Storm 840 Imager (Molecular Dynamics). The amount of GST-C21-bound Rho-GTP was normalized to the total amount of Rho in the cell lysate.

Visualization of F-actin fibers. CPAE cells (either nontransfected cells stimulated with 25% HTS or 2.5 U/ml thrombin or transfected cells expressing the constitutively active isoform of Gα13, RhoA, or Rho kinase) were fixed for 10 min at room temperature in 3.7% formaldehyde in PBS and permeabilized in 0.2% Triton X-100 in PBS for 4 min. After being blocked with 10% FCS in PBS for 30 min, cells were incubated with 1 unit of rhodamine-phalloidin (Molecular Probes) diluted in PBS with 1% FCS per coverslip for 20 min at room temperature. Cells were rinsed twice in PBS after each incubation. Finally, the cells were washed twice with water. To prevent photobleaching, cells were mounted in Vectashield (Vector Laboratories). Labeled cells were observed with 1) a Nikon Optiphot-2 microscope using green excitation for visualization of rhodamine staining and blue excitation to identify the green fluorescent cells, or 2) a Leica SP-2 laser scan confocal microscope.

Quantification of F-actin. The cellular F-actin content was estimated essentially as described previously (27), except that cells were attached to multwell plastic dishes. Cells were seeded at 90,000 cells/well and after stimulation with 25% HTS or 2.5 U/ml thrombin fixed in 3.7% paraformaldehyde in TBS. The cells were washed in 0.1% saponin buffer (in mM: 10 MOPS pH 6.9, 5.5 EGTA, 20 K2HPO4, 1.95 MgSO4, and 0.1% wt/vol saponin) for permeabilization and incubated with 200 μl of 0.33 μM rhodamine-phalloidin diluted in 0.1% saponin buffer. After 1 h at room temperature, cells were washed twice in MOPS buffer (in mM: 10 MOPS pH 6.9, 5.5 EGTA, 20 K2HPO4, and 1.95 MgSO4) and incubated in 1 ml methanol for 30 min to extract the rhodamine-phalloidin. The solution was transferred to a cuvette, and methanol was added to a final volume of 2.5 ml. Rhodamine fluorescence was measured spectrofluorometrically at 576 nm after excitation at 540 nm using a PTI RatioMaster spectrophotometer. As a control, identical samples were incubated as described above, except in the presence of a 100-fold excess of unlabeled phalloidin (Molecular Probes). The fluorescence measured from these samples was <4% of that obtained under standard conditions. Data for swollen and thrombin-stimulated cells are presented as the 576-nm emission (after subtraction of a methanol blank) normalized to the blank-subtracted rhodamine signal of nonstimulated control cells (relative scale). The assay was verified to be linear over a range of at least 60,000–100,000 cells per well (data not shown).

Biochemical effect of C2IN-C3 toxin on Caco-2 cells. Caco-2 cells were incubated with C2IN-C3 toxin by adding 200 ng C2I1 and 200 ng C2IN-C3/ml culture medium. After overnight incubation, cells were lysed in a 1% Triton buffer containing (in mM) 25 Tris, 100 NaCl, 90 mannitol, 2 DTT, 1 mM EGTA, 1% Nonidet P-40, 2 mM PMSF, 0.4 mM sodium pervanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin.

CPAE cells were incubated with the pGEX3X vector containing the glutathione S-transferase (GST)-C21 construct (a kind gift of Dr. J. G. Collard) that encodes the Rho-binding domain of Rhotekin (a Rho effector protein) fused to GST. GST-C21 protein synthesis was induced with 0.1 mM isopropyl β-D-thiogalactoside. After 12 h, the culture was centrifuged (1,000 C, 5 min), and aliquots of the supernatant were used for 12% SDS-PAGE. After blotting, Rho was detected with a monoclonal antibody (Santa Cruz Biotechnology sc-417; diluted 1:2,000) and a secondary alkaline-phosphatase-conjugated goat anti-mouse IgG (diluted 1:7,000) as described previously (26).

Data analysis. Analysis of electrophysiological data was performed using the WinASCD software (Guy Droogmans, Laboratorium voor Fysiologie, KU Leuven). The Origin software package version 6.0 (Microcal Software, Northampton, MA) was used for statistical analysis and graphical presentation of the data.

Time courses of the whole cell current were obtained by plotting the current at −100 mV and respectively at +50 mV (Caco-2) or +100 mV (CPAE). Current-voltage relations were obtained from the currents measured during the linear voltage ramp. Difference currents (ICl,swell) were calculated by subtracting the basal current under isotonic conditions from the maximal current during hypotonic stimulation.

Pooled data are given as means ± SE from n cells. Significance between two data sets was tested using Student’s unpaired t-test. One-way ANOVA was used to determine statistical differences of three or more data sets. Differences were considered significant at the level of P < 0.05.

RESULTS

Transient expression of constitutively active Gα13, RhoA, or Rho kinase does not affect ICl,swell in CPAE cells. In a first series of experiments, we investigated the effect of constitutively active isoforms of Gα13 (mutation Gln226Leu; see Ref. 16), RhoA (mutation Glu262Leu; see Ref. 16), RhoA (mutation Gin63Glu; see Ref. 30) or Rho kinase (CAT: Rho kinase containing only the catalytic domain; see Ref. 2) on...
In transiently transfected CPAE cells. Under isotonic conditions, the basal membrane current of control CPAE cells had a mean density of 13.4 ± 2.8 pA/pF at +100 mV (n = 31; Fig. 1). CPAE cells transfected with RhoA Gln63Glu developed slightly larger basal membrane currents during isotonicity with a mean density of 26 ± 5.3 pA/pF (n = 15) at +100 mV (Fig. 1). However, this value was not significantly different from that in control cells. Similarly, the basal membrane current in CPAE cells transfected with either Ga13 Gln226Leu or Rho kinase-CAT did not differ significantly from that in wild-type CPAE cells: the basal membrane current had a mean density of, respectively, 18.4 ± 3.4 pA/pF (n = 40) and 16.8 ± 5.54 pA/pF (n = 16) at +100 mV (Fig. 1).

When exposed to a 25% HTS, wild-type CPAE cells developed large \( I_{\text{Cl,swell}} \) currents with a mean current density of 85.5 ± 9.2 pA/pF at +100 mV (n = 24; Fig. 1). The hypotonicity-induced membrane currents in CPAE cells transfected with either Ga13 Gln226Leu, RhoA Gln63Glu, or Rho kinase-CAT had a similar \( I_{\text{Cl,swell}} \) phenotype, of which the mean current density did not differ significantly from the control value: 72.3 ± 9.2 pA/pF (n = 27) for Ga13 Gln226Leu, 79.1 ± 7.3 pA/pF (n = 11) for RhoA Gln63Glu, and 77.2 ± 4.8 pA/pF (n = 13) for Rho kinase-CAT, in each case at +100 mV (Fig. 1).

To control for the expression of the constitutively active isoforms of Ga13, RhoA, and Rho kinase, we stained actin filaments with rhodamine-labeled phalloidin in transiently transfected CPAE cells. Figure 2 clearly shows the formation of actin stress fibers in transfected cells, which were identified by the expression of green fluorescence protein (GFP). As reported by Watanabe et al. (42), the Rho kinase-CAT-induced stress fibers had a stellate appearance around a central focus (Fig. 2F), whereas Ga13 Gln226Leu- and RhoA Gln63Glu-induced stress fibers ran in parallel bundles throughout the whole cell (Fig. 2, B and D). Control experiments in which CPAE cells were transfected with GFP alone indicated that expression of GFP had no effect on the formation or distribution of stress fibers (Fig. 2, G and H). Thus, although we could clearly demonstrate activation of the Rho pathway in the transfected CPAE cells, there was no spontaneous VRAC activity under isotonic conditions and no potentiation after hypotonic cell swelling by 25% HTS.

Hypotonic cell swelling by 25% HTS does not activate the Rho pathway in CPAE cells. In a complementary series of experiments, we investigated whether hypotonic cell swelling triggers activation of the Rho pathway. To measure Rho activation, we used a pull-down assay in which the active Rho-GTP, but not the inactive Rho-GDP, is bound to a GST fusion protein containing the Rho binding domain of Rhotekin (39). Rho-GTP formation was monitored during a 4-min 25% HTS, which in CPAE cells is sufficiently long to fully activate VRACs, since we have previously shown that it takes between 60 and 90 s for a 25% HTS to half-maximally activate VRACs in CPAE cells (25, 36). As shown in Fig. 3, the ratio of active Rho-GTP to total Rho did not change significantly during hypotonic swelling of CPAE cells. In contrast, stimulation of CPAE cells with thrombin (5 U/ml) induced the formation of Rho-GTP (Fig. 3), as has also been observed for human umbilical vein endothelial cells (39).

We also checked the downstream activity of the Rho pathway by assessing the formation of F-actin stress fibers before and during cell swelling (10 min, 25% HTS). Cellular F-actin content was visualized with a fluorescence microscope and a laser scanning confocal microscope. Net cellular F-actin content was quantified spectrofluorometrically. Consistent with the lack of Rho activation, there was no obvious change in the pattern or amount of F-actin stress fibers in swollen vs. control cells, neither as evaluated from the fluorescence microscope pictures (Fig. 4) nor as seen in the confocal microscope (data not shown). There was also no significant change in the net cellular F-actin content during cell swelling (Fig. 4, top right). Again, formation of stress fibers was easily demonstrated in thrombin-stimulated cells, and a significant increase in the net amount of F-actin stress fibers was measured by 10.2 ± 0.32.246 on November 6, 2017 http://ajpcell.physiology.org/ Downloaded from

![Fig. 1. Expression of either Ga13 Gln226Leu, RhoA Gln63Glu, or Rho kinase-CAT in calf pulmonary artery endothelial (CPAE) cells does not potentiate the swelling-induced chloride current (\( I_{\text{Cl,swell}} \)). Membrane currents under isotonic (ISO; total membrane current) and hypotonic (HTS; \( I_{\text{Cl,swell}} \) difference current) conditions were measured in control CPAE cells and CPAE cells transiently transfected with, respectively, Ga13 Gln226Leu, RhoA Gln63Glu, or Rho kinase-CAT, as indicated. Current densities (means ± SE) at +100 mV are plotted for the different conditions. ANOVA did not reveal statistical differences between the isotonic currents or between the hypotonic currents.](http://ajpcell.physiology.org/DownloadedFrom)
cellular F-actin content was measured after exposure to thrombin (Fig. 4). To conclude, we could not detect any biochemical or morphological alterations in the Rho pathway during cell swelling, indicating that cell swelling does not activate the Rho pathway in CPAE cells.

The Rho pathway is not required for activation of VRACs in caveolin-1 transfected Caco-2 cells. In a second part, we addressed the question whether a functional Rho pathway is a general requirement for VRAC activation as is observed in CPAE cells (25, 26) or, alternatively, whether VRAC activation can occur independently of the Rho pathway. These experiments were performed in Caco-2, a colon carcinoma cell line that does not express endogenous caveolin-1 (37). We have previously shown that a hypotonic stimulus is unable to activate VRACs in wild-type Caco-2 cells, but that expression of caveolin-1 restores efficient VRAC activation (37). We first confirmed by Western blotting that RhoA is expressed in Caco-2 cells (data not shown; see also Fig. 6D, inset), as has also been demonstrated by others (11). Subsequently, we investigated whether manipulating the Rho pathway, either by stimulation with GTPγS or by inhibition with the C2IN-C3 fusion toxin, modulated \( I_{Cl,swell} \) in wild-type and caveolin-1 transfected Caco-2 cells.

As shown previously, wild-type Caco-2 cells did not respond to a 25% HTS: the mean current density of the
HTS-induced current was 2.8 ± 1.6 pA/pF at +50 mV (n = 13; see Fig. 5A for pooled data). Similarly, intracellular application of GTPγS (100 μM) via the patch pipette under isotonic conditions was unable to trigger VRACs (see Fig. 5A for pooled data). At the onset of the GTPγS application, there was even a slight decrease of the basal membrane current before stabilizing. The change in basal current amounted to −2.6 ± 1.0 pA/pF (n = 14) at +50 mV. In control experiments performed on the same day and with the same batch of pipette solution, we were able to transiently activate VRAC in CPAE cells (data not shown), thereby validating the GTPγS solution. We then examined VRAC activity in Caco-2 cells that had been transiently transfected with a caveolin-1 expression vector. In caveolin-1-expressing Caco-2 cells, intracellular application of GTPγS (100 μM) had no significant effect on the membrane current under isotonic conditions (Fig. 5B). The mean current density of the GTPγS-induced current was 0.3 ± 1.4 pA/pF (n = 25) at +50 mV (Fig. 5A). In contrast, subsequent exposure of caveolin-1-expressing Caco-2 cells to a 25% HTS resulted in a significant increase in membrane current (Fig. 5B). The mean current density of the HTS-induced current amounted to 21.3 ± 3.5 pA/pF (n = 24) at +50 mV (Fig. 5A). The time course and current density (Fig. 5, B and C) of the HTS-induced current in GTPγS-perfused, caveolin-1-expressing Caco-2 cells were comparable to the I_{Cl,swell} properties previously characterized in caveolin-1-expressing Caco-2 cells (37). Thus, in contrast to CPAE cells, GTPγS is unable to activate VRACs in caveolin-1-expressing Caco-2 cells.

The C. limosum C3 exoenzyme specifically inactivates Rho by ADP-ribosylation (15). Because the C3 exoenzyme does not penetrate the cell, we used a C2IN-C3 fusion toxin in which the C3 exoenzyme is fused to the amino-terminal part of C. botulinum C2I. Simultaneous incubation of C2IN-C3 with C2I results in efficient cellular uptake of the C3 toxin (4, 26). We have previously shown that pretreatment of CPAE cells with the C2IN-C3/C2II cocktail significantly impaired the activation of I_{Cl,swell} in response to either a 25% HTS or the intracellular perfusion of GTPγS, whereas the individual application of either C3 or C2II did not affect VRACs (26). We used the same protocol to incubate caveolin-1-expressing Caco-2 cells with the C2IN-C3/C2II toxin mix. Application of a 25% HTS generated a normal I_{Cl,swell} response in caveolin-1-expressing Caco-2 cells whether or not they had been pretreated with C2IN-C3/C2II (Fig. 6, A and D); the mean current density of I_{Cl,swell} at +50 mV was 31.6 ± 5.6 pA/pF (n = 35) for untreated Caco-2 cells vs. 50.7 ± 13.2 pA/pF (n = 13) for toxin-treated cells (no significant difference). The phenotypical properties (Fig. 6, B and C) of the HTS-induced current in C2IN-C3/C2II treated, caveolin-1-expressing Caco-2 cells were identical to the I_{Cl,swell} properties previously characterized in caveolin-1-expressing Caco-2 cells, except for the degree of inactivation, which was less pronounced in the present series of experiments (37). The biological activity of C2IN-C3/C2II was verified in CPAE cells. Pretreatment of these cells with C2IN-C3/C2II resulted in a significant downregulation of I_{Cl,swell} (see Fig. 6D). We also tested whether Rho was ADP-ribosy-
Hypotonic cell swelling does not change the F-actin content or organization in CPAE cells. CPAE cells were stimulated with 25% HTS (left) or thrombin (2.5 U/ml; right) for 0, 2, 4, 5, or 10 min as indicated. After fixation, F-actin filaments were stained with rhodamine-labeled phalloidin and visualized by fluorescence microscopy. F-actin formation was quantified by measuring rhodamine fluorescence as described in MATERIALS AND METHODS. The graph (top right) shows the F-actin content in HTS- or thrombin-treated CPAE cells normalized to the F-actin content in control cells (relative scale). Data points represent means ± SE (n = 3). As shown, 25% HTS has no effect on the distribution or amount of actin fibers, whereas thrombin treatment clearly increases actin filaments. All cells were visualized at the same magnification.
lated in C2IN-C3/C2II-pretreated Caco-2 cells. As shown in the inset in Fig. 6D, pretreatment of Caco-2 cells with C2IN-C3/C2II resulted in a slower migration of Rho due to ADP-ribosylation compared with non-pretreated cells, indicating that the toxin had indeed penetrated the cell and modified Rho (5).

DISCUSSION

We have previously shown that activation of VRACs in vascular endothelial cells (CPAE) requires a functional Rho/Rho kinase/myosin light chain phosphorylation pathway, since inhibition of Rho, Rho kinase, or myosin light chain kinase abrogates the swelling-induced activation of VRACs. Yet, in this study we show that activation of the Rho pathway by constitutively active isoforms of Gα13, Rho, or Rho kinase has no effect on VRACs either in isotonic or in hypotonic conditions. Importantly, we did not observe spontaneous activation of VRACs in control conditions or potentiation during cell swelling. In a complementary series of experiments we examined whether cell swelling would activate the Rho pathway, but there was no indication that this pathway was turned on by cell swelling. These data are consistent with previous observations on the effect of thrombin on VRACs: thrombin is unable to trigger VRACs under isotonic conditions (21), despite it being a potent stimulator of the Rho pathway in endothelial cells (9, 39). In view of the inability of the Rho pathway to activate VRACs and the lack of activation of this pathway during hypotonic cell swelling, we conclude that the Rho pathway does not play a causal role in swelling-induced activation of VRACs.

Fig. 5. Caveolin-1 transfected Caco-2 cells react to hypotonic solution but are unresponsive to guanosine 5′-O-(3-thiotriphosphate) (GTPγS). A: pooled data for the membrane currents during intracellular application of GTPγS in control and caveolin-1-transfected Caco-2 cells under isotonic and hypotonic conditions. ISO GTPγS represents the current density (means ± SE) at +50 mV of the GTPγS-induced current under isotonic conditions. HTS GTPγS represents the current density at +50 mV of I_{Cl,swell} (25% HTS) during GTPγS perfusion. Control, nontransfected cells; +cav-1, cells transiently transfected with caveolin-1 expression vector; ISO, isotonic condition; HTS, 25% hypotonic condition; GTPγS was applied via the patch pipette and hence present in all conditions as indicated. In nontransfected cells, GTPγS induces a slight decrease of the membrane current during isotonic perfusion. B: time course of the membrane current at +100 mV (top trace) and at −100 mV (bottom trace) in a representative caveolin-1 transfected Caco-2 cell. Intracellular perfusion of 100 μM GTPγS during isotonicity does not induce activation of a membrane current, whereas a subsequent 25% HTS triggers a pronounced increase in membrane current, which is reversible on returning to isotonic conditions. Traces start immediately after obtaining the whole cell configuration.

C: family of current traces of a caveolin-1 transfected Caco-2 cell during voltage steps applied at the times indicated by the asterisks in B during isotonicity (ISO) and hypotonicity (HTS). D: current-voltage curves taken at the times marked by the filled symbols (a and b) in B.
can be reconciled with the previously established requirement for a functional Rho pathway by postulating a permissive effect for the Rho pathway, i.e., Rho pathway activity is required for but does not trigger opening of VRACs during hypotonic cell swelling. One implication of such a permissive effect would be that the Rho pathway could help to sensitize the cell to hypotonic stimuli. Indeed, we have previously observed that thrombin potentiates VRACs at mild hypotonic stimulation (13%) but less so at stronger hypotonic stimuli (28% HTS) (21). In addition, it would explain why thrombin or a myosin light chain phosphatase inhibitory peptide (NIPP1191–210) potentiates VRACs once the channels have been preactivated by a hypotonic stimulus, although they exert no effect on VRACs under isotonic conditions (21, 25). What could be the molecular basis for such a permissive effect? As reviewed by Janmey (14), the cytoskeleton forms a three-dimensional network that not only determines the mechanical properties of the cell but also forms an extended scaffold onto which regulatory and signaling proteins can bind. In this context, the Rho pathway could facilitate VRAC activation by promoting the formation of actin filaments that would serve as a platform on which proteins that participate in the VRAC activation cascade assemble.

Although we were unable to detect alterations in Rho activity, F-actin stress fibers, or net cellular F-actin content during cell swelling, cytoskeletal rearrangements during hypotonic swelling have been described before (17, 19, 23, 27, 35). Pedersen et al. (27) and Levitan et al. (19) reported a disruption of the cortical F-actin cytoskeleton in, respectively, Ehrlich ascites tumor cells and B-lymphocytes, whereas formation of membrane ruffles due to cortical actin polymerization was observed in intestinal 407 cells (35) and C6 glioma cells (23). Koyama et al. (17) observed a transient formation of F-actin stress fibers during hypotonic swelling of bovine aortic endothelial cells. However, it was concluded that the observed cytoskeletal rearrangements during hypotonic cell swelling were not critical in initiating regulatory transport processes (17, 19, 23).

A second finding of this study is the variable requirement for a functional Rho pathway. In CPAE cells, intracellular application of GTPγS triggers a transient outwardly rectifying chloride current that is phenotypically identical to \( I_{\text{Cl,swell}} \) (41). This effect of GTPγS is
mediated via Rho and Rho kinase, since inhibition of Rho with the C3 exoenzyme or of Rho kinase with Y-27632 abolishes the GTPγS-induced current in endothelial cells (26). More recently, Koyama et al. (17) showed that inhibition of Rho with C3 toxin or of Rho kinase with Y-27632 also reduced the swelling-induced ATP release by bovine aortic endothelial cells. Thus, in vascular endothelial cells, swelling-induced responses seem critically dependent on a functional Rho system. A similar conclusion can be drawn for N1E115 neuroblastoma cells in which blockade of Rho by the C. difficile toxin B also reduces I_{Cl\text{swell}} (10). In contrast, caveolin-1-expressing Caco-2 cells were unresponsive to GTPγS despite normal VRAC activation by a hypotonic stimulus. Furthermore, C3 exoenzyme pretreatment did not affect the HTS-triggered activation of VRACs. First, this is consistent with the previous conclusion that the Rho pathway does not transmit the primary activating signal during cell swelling. Second, it also suggests that the permissive effect of the Rho pathway is either dispensable in Caco-2 cells and/or that there are alternative structures that can take over the permissive role of the Rho pathway. This conclusion is compatible with the previously reported failure of GTPγS to activate I_{Cl\text{swell}} in Xenopus oocytes (1) or in C6 glia cells (34). It therefore seems that, at least in these experimental systems, Rho is not required for swelling-induced activation of VRACs.

In summary, the present data, in combination with our previous observations on VRACs in CPAE cells, allow us to delineate more precisely the contribution of the Rho pathway to hypotonicity-induced activation of VRACs. An intact Rho/Rho kinase/myosin light chain phosphorylation pathway is required for VRAC activation in CPAE cells. However, it does not transmit the activator signal that opens VRACs during cell swelling. We therefore propose that the Rho pathway fulfills a permissive role, but not a causal role, during VRAC activation in CPAE cells.

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