Importance of cytoskeletal elements in volume regulatory responses of trout hepatocytes

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The role of cytoskeletal elements in volume regulation was studied in trout hepatocytes by investigating changes in F-actin distribution during anisotonic exposure and assessing the impact of cytoskeleton disruption on volume regulatory responses. Hypotonic challenge caused a significant decrease in the ratio of cortical to cytoplasmic F-actin, whereas this ratio was unaffected in hypertonic saline. Disruption of microfilaments with cytochalasin B (CB) or cytochalasin D significantly slowed volume recovery following hypo- and hypertonic exposure in both attached and suspended cells. The decrease of net proton release and the intracellular acidification elicited by hypotonicity were unaltered by CB, whereas the increase of proton release in hypertonic saline was dramatically reduced. Because amiloride almost completely blocked the hypertonic increase of proton release and cytoskeleton disruption diminished the associated increase of intracellular pH (pHi), we suggest that F-actin disruption affected Na+/H+ exchanger activity. In line with this, pHi recovery after an ammonium prepulse was significantly inhibited in CB-treated cells. The increase of cytosolic Na+ under hypertonic conditions was not diminished but, rather, enhanced by F-actin disruption, presumably due to inhibited Na+-K+-ATPase activity and stimulated Na+ channel activity. The elevation of cytosolic Ca2+ in hypertonic medium was significantly reduced by CB. Altogether, our results indicate that the F-actin network is of crucial importance in the cellular responses to anisotonic conditions, possibly via interaction with the activity of ion transporters and with signalling cascades responsible for their activation. Disruption of microtubules with colchicine had no effect on any of the parameters investigated.

Oncorhynchus mykiss; F-actin; microtubule; acid secretion; intracellular pH; intracellular sodium; intracellular free calcium

THE REGULATION OF CELL VOLUME is of pivotal importance for the maintenance of structural integrity, a constant intracellular milieu, and, ultimately, cell survival. Thus, after volume changes evoked by anisotonic conditions, most cells are capable of restoring their cell volume by activating various mechanisms, mediating a net loss or uptake of osmolytes as required (2). Particularly in mammalian cells, the various transporters involved in these volume regulatory responses have been well characterized over the past decades (64), but there is still a considerable gap in our knowledge of the factors controlling and regulating their activity. One aspect that has received increasing interest during the last years is the role of cytoskeletal elements, including microfilaments, microtubules, and associated proteins (53). Concerning the actin-based cytoskeleton, several studies have shown that anisotonic changes of cell volume are associated with significant changes of cellular F-actin content and/or distribution (29, 50, 54, 66). Conversely, disruption of F-actin has been shown to delay or even totally inhibit volume regulation in a number of cell models (19, 44, 54). Similar results have been reported with regard to the microtubule network, although this has been less thoroughly studied (15, 19, 28). The mechanistic basis of these effects is not yet fully understood, but various reports suggest that cytoskeleton elements may directly control the activity of a number of transporters (40, 43, 48) or participate in the process of vesicle-mediated insertion and retrieval of transport proteins (53).

Based on these results, the importance of the cytoskeleton as a factor involved in volume regulatory responses is widely accepted, but there also exist a number of studies in which cytoskeletal disrupting agents had no effect on volume regulation (12, 25, 42) or in which anisotonic volume changes did not elicit quantitative changes in the abundance of cytoskeletal elements (29). Although these differences may be partly attributed to differences in the experimental approaches applied, there also is some evidence that they may reflect genuine cell type-specific differences, at least for mammalian cells. With regard to nonmammalian cells, in addition to older studies on frog gallbladder (19), shark rectal gland (35, 66), and vesicles from Necturus enterocytes (16), there also is a recent report on eel intestinal cells (45) demonstrating a close link between the cytoskeleton and volume regulation. All of these cell types studied are specialized epithelial cells exposed to severe anisotonic challenges on a regular basis. However, to our knowledge, there is no study yet available on isolated hepatocytes from lower vertebrates, such as rainbow trout hepatocytes, which may be taken as a representative model for epithelial cells that are located in a more constant osmotic environment because they are not facing the environmental water. Furthermore, the trout hepatocyte is a well-established model system for the study of lower vertebrate cell function, for which several studies have investigated the capability of volume recovery under hypo- and hypertonic conditions and the transport mechanisms involved in volume regulation (5, 6, 20, 37).
49, 52). However, information concerning the interaction between cytoskeleton and volume regulation of these cells is missing so far. It is therefore not known whether trout hepatocytes behave similarly to their mammalian counterparts in this regard or if they display deviating response patterns, as recently has been observed with respect to the metabolic alterations elicited by anisotonic conditions (37).

In the present study we therefore aimed at addressing the role of cytoskeletal elements for volume regulation in trout hepatocytes. The goals of our study were to evaluate 1) alterations of cytoskeletal elements, specifically of the actin-based cytoskeleton, during anisotonic exposure of the hepatocytes; 2) the impact of cytochalasin B (CB) and D (CD), F-actin disrupting agents, on volume regulation in anisotonic conditions; and 3) the effect of cytoskeletal disruption on ion movements associated with volume regulatory responses. In addition, we studied the effect of the microtubule disrupting agent colchicine on some parameters of interest to evaluate the possible importance of microtubules in volume regulation.

MATERIALS AND METHODS

Materials. Collagenase (type VIII), bovine serum albumin (BSA), fetal calf serum (FCS), ionophores, antibiotics, CB, CD, colchicine, amiloride, 4-acetamido-4′-isothiocyano-Stilbene-2,2′-disulfonic acid (SITS), latrunculin B, ouabain, and poly-t-lysine all were purchased from Sigma (Deisenhofen, Germany). The acetoxyethyl esters (AM) of 2,7′-bis-(2-carboxypropyl)-5(6)-carboxyfluorescein (BPCF), fura-2, calcine, and sodium-binding benzofuran isothiphen (SBFI) and Alexa Fluor phallidin were from Molecular Probes (Leiden, Netherlands). Cariporide was a kind gift from Sanofi-Aventis Pharma (Vienna, Austria). Leibovitz L-15 medium was bought from Invitrogen. The mouse anti β-tubulin monoclonal antibody (ABD 3408) was obtained from Chemicon, the TRITC-labeled anti mouse secondary antibody was from DAKO, and Vectashield mounting medium was from Vector Laboratories. All other chemicals were of analytical grade and were purchased from local suppliers.

Preparation of hepatocytes and cell culture. Rainbow trout, Oncorhynchus mykiss, were obtained from a local hatchery. Animals were acclimated at 15°C in 200-liter aquaria and were daily fed ad libitum with trout pellets. Trout were killed with a sharp blow on the head and subsequent transection of the spinal cord. Maintenance and use of experimental animals were in accordance with the Austrian federal law for care and use of laboratory animals. Hepatocytes were isolated using a method based on collagenase digestion and differential centrifugation, as described previously (36). After cell isolation, hepatocytes were suspended in standard saline (for composition, Experimental media) with 1% BSA and were left to recover for 1 h in a shaking water bath thermostated to 19°C, which also was the temperature used during the experiments. Cells were then counted, and viability was determined using the Trypan blue exclusion method.

Experimental media. The standard incubation medium with an osmolarity of 284 mosM consisted of (in mM) 10 HEPES, 136.9, NaCl, 5.4 KCl, 1 MgSO₄, 0.33 NaH₂PO₄, 0.44 KH₂PO₄, 1.5 NaHCO₃, 1.5 CaCl₂, and 5 glucose, pH 7.6, at 19°C. Hypotonic medium with an osmolarity of 166 mosM consisted of a mixture of equal volumes of standard saline and the same medium without NaCl (0.58× isomolarity). Similarly, a mixture of standard saline and saline containing an additional 200 mM NaCl was used to create hypertonic conditions, yielding an osmolarity of 465 mosM (1.6× isomolarity). Medium osmolarity was assessed by freezing point depression using a Knaus Semi-Micro osmometer (Berlin, Germany).

Experimental exposure, fixation, and staining of hepatocytes for confocal microscopy. Primary cell cultures were carefully washed with standard saline and then incubated in this medium for at least 1 h. Subsequently, cells were exposed to one of the following treatments:

1) Hepatocytes were incubated with 2 μM CB in standard saline for 30 min or, for comparative reasons, with 2 μM of the analog CD for 15 min, or with 2 μM latrunculin B for 15 min. The latter compound leads to a disruption of F-actin by a mechanism differing from that of the cytochalasins. As established in preliminary studies and shown below, these concentrations of CB, CD, and latrunculin B were found suitable to cause a visible disruption of the F-actin network.

2) Cells were incubated with 100 μM colchicine in isotonic medium for 30 min, which produced a decrease of visible microtubule elements.

3) Cells were exposed to hypotonic conditions for 5 and 30 min.

4) Hepatocytes were incubated in hypertonic saline for 5 and 30 min. At the end of the respective incubation period, hepatocytes were washed twice with phosphate-buffered saline (PBS, pH 7.6) and fixed for 60 min at room temperature with 4% paraformaldehyde in PBS. For confocal visualization of actin and tubulin, cells were permeabilized by treatment with PBS containing 0.1% Triton X-100 and then blocked over 1 h in PBS containing 0.1% Triton X-100, 1% BSA, and 1% FCS. Subsequently, cells were incubated overnight in a humidified dark chamber at +4°C with a 1:200 dilution of mouse anti β-tubulin monoclonal antibody and a 1:150 dilution of Alexa Fluor phallidin. After three washes in PBS with 0.1% Triton X, the coverslips were further incubated in the dark chamber for 1 h in the presence of TRITC-labeled anti mouse secondary antibody (1:150) at room temperature, and finally, after additional washings, the slides were covered with mounting medium and fixed on object slides. For the analysis of actin only, the overnight incubation step, as well as incubation with secondary antibody, was omitted. Cells were visualized with a confocal laser scanning fluorescence microscope (LSM 510; Zeiss) with excitation and emission wavelengths of 488 and 505–550 nm for Alexa Fluor phallidin and of 543 and >585 nm for TRITC. Three-dimensional reconstruction images of the hepatocytes were created from serial 1-μm optical slices acquired using the LSM 510 software (version 3.2; Zeiss).

F-actin quantification. Quantification of relative F-actin distribution was made using confocal laser scanning microscopy by applying the method described by Erickson et al. (17). This method is based on the observation that, similar to chondrocytes (17), F-actin of resting hepatocytes is predominantly localized near the plasma membrane and that it is this cortical actin that is mainly affected by cell swelling or shrinkage. Accordingly, the ratio of cortical actin fluorescence to that of cytoplasmic actin was used to be a sensitive measure to quantify changes in the actin-cytoskeleton. By determining the ratio, this method also circumvents the problem of variations in staining efficiency between individual cell cultures. For these measurements, F-actin was labeled with the fluorescence phallidin derivative in fixed cells as described above. Images for these measurements were captured using a plan Neofluar ×40/1.30 oil objective lens with a large pinhole setting resulting in an optical slice thickness of 7 μm. With the use of Zeiss software, a linear intensity profile was laid through the equatorial plane of individual cells, and the average of the peak intensities measured near the cell membrane (Iₘembrane) and that of the cytoplasmic region between these two points was determined (Iₘembrane - Iₘembrane). F-actin distribution was then calculated according to the following formula: actin ratio = (Iₘembrane - Iₘembrane)/(Iₘembrane + Iₘembrane).
CaCl$_2$, and 5 mM glucose. The pH of all solutions was adjusted to a

**Cell water volume.** Cell water volume of cultured hepatocytes was estimated using epifluorescence microscopy according to the method of Altamirano et al. (1) and modified by Espelt et al. (18). In brief, coverslips with attached cells were mounted in a chamber filled with isometric medium and placed on the stage of a Nikon TE-200 inverted epifluorescence microscope. Hepatocytes were then loaded with 2 μM calcein AM for 45–60 min, followed by a period of 1 h during which the loading solution was washed out with isometric medium superflusified at a rate of 2 ml/min. Subsequent medium changes were conducted by hand. Estimates of cell water volume changes were obtained from changes in fluorescence intensity recorded from a small pinhole region of each cell as detected through a 500-nm long-pass (LP) dichroic mirror and a 515-nm LP barrier filter after exciting calcein though a 470-nm center wavelength excitation filter. Under the conditions applied, changes in calcein fluorescence are proportional to its concentration and are therefore inversely related to alterations of cell water volume (18). Calibrations were conducted before each measurement by exposing cells to hypo-, iso-, and hypertonic solutions of known osmolarity. Osmotic changes induced by the calibration solutions were selected so that no volume regulatory response of the cell

**Volume changes of suspended cells** were determined by electronic cell sizing on a Coulter Counter Multisizer II, with an aperture of 100 μm. Aliquots of cell suspensions were diluted into 20 ml of iso- or anisotonic media as given above, and at specified time points, ~20,000 cells were measured and their mean volume compared with that of cells at time t = 0.

For quantitative comparison of the extent of regulatory volume changes (RVC) observed in response to anisotonic exposure of the cells, the initial rate of cell volume recovery (rRVC) and the relative volume change 20 min (RVC20) or 40 min (RVC40) after maximum cell swelling or shrinkage (Vrmax and Vrmin, respectively) were calculated. rRVC was obtained by fitting data of relative volume change vs. time by the second-order polynomial $A_t + rRVC t + Ct^2$, with the first derivative of this function at time 0 corresponding to rRVC. RVC20 and RVC40 were calculated as $(Vr_{max} - Vr)/Vr_{max} - 1$ for hypotonic exposure and $(Vr_{min} - Vr)/(Vr_{min} - 1)$ for hypertonic exposure, where Vr corresponds to the relative cell volume at the respective time after maximum volume change.

**Proton secretion.** Proton secretion of hepatocytes was assessed with a cytosensor microphysiometer (Molecular Devices) by measuring the rate of acidification of external medium as described (55), with modifications (39). To this end, we embedded cells (0.45 × 10⁶ hepatocytes) in 1.5% low-melting-point agarose on the polycarbonate membrane of capsule cups and mounted them in the sensor chamber of the cytosensor microphysiometer. In this chamber, the cells were superfused via one of two fluid outlets that may be rapidly switched using an electromagnetic valve. The flow cycle was set to 3 min, with 2.5 min of constant superfusion and a 30-s flow-off period. During this time, the protons released by the hepatocytes accumulated in the sensor chamber, and this was registered via a light-addressable potentiometric sensor. The rate of acidification was then calculated from the slope of a line fitted to the sensor data. The saline used was the same as those used for SBFI AM (15 μM; 15 mM stock dissolved in DMSO and 20% Pluronic acid). The loading solution was then washed out with isometric medium, and the cells were incubated for at least another 30 min. Measurements of [Na⁺] were conducted on the microscope setup described in Intracellular pH (Zeiss Axiovert) with excitation set to 340 and 380 nm and emission measured above 510 nm. Calibrations were performed using the method of Harootunian et al. (27), with minor modifications. In short, the bathing saline was changed to a Na⁺-free gluconate solution consisting of 136.9 mM K-gluconate, 10 mM HEPES, 5.4 mM KCl, 1 mM MgSO₄, 0.77 mM KH₂PO₄, 5 mM K₂HPO₄, 1.5 mM CaCl₂, and 5 mM glucose, containing the ionophores gramicidin D (4 μM), monensin (10 μM), and nigericin (10 μM), with pH 7.6 adjusted with KOH. By substitution of Na-glucinate for K-gluconate, Na⁺ concentration was gradually increased from 0 to 20 and 50 mM, and a standard curve relating the ratio (340/380) to [Na⁺] was established.

**Rb⁺ uptake.** Rates of Rb⁺ uptake, serving as a measure of transmembrane K⁺ fluxes, were determined in control cells and in hepatocytes preincubated with 2 μM CB for 30 min. Rb⁺ fluxes were determined both in the presence of 1 mM ouabain, a selective inhibitor of Na⁺-K⁺-ATPase activity, and in the absence of the inhibitor. The method used for Rb⁺ uptake measurement has been described in detail previously (36).

**Cytosolic free Ca²⁺.** The fluorescent indicator dye fura-2 AM was used to measure cytosolic free calcium concentration ([Ca²⁺]), using the microscope setup described in Intracellular pH (Zeiss Axiovert). Images of fura-2-loaded cells were captured every 60 s, with settings identical to those given for SBFI applied. Protocols for anisotonic exposure were the same as those used for pH measurements. Fluorescence ratios were stored, and absolute [Ca²⁺], were calculated using the formula given by Grynkiewicz et al. (24), with a dissociation constant (Kd) of 680 nM as determined previously for our experimental setup using a commercial calibration kit (Molecular Probes) (37). After each experiment, a maximum fluorescence ratio, obtained by addition of 4.5 mM CaCl₂, and a minimum ratio, obtained after addition of 20 mM EGTA, both in the

**Intracellular pH.** Intracellular pH (pHᵢ) of attached individual hepatocytes was measured using the pH-sensitive fluorescent dye BCP, as described previously (38). Cells loaded with the fluorophore were mounted in a stainless steel measuring chamber and fixed on an inverted fluorescence microscope (Axiovert, Zeiss) equipped with a ×40 UV objective. Fluorescence was determined at 60-s intervals with excitation at 440 and 490 nm and emission detected above 510 nm. Hyper- and hypotonic conditions were created by exchanging 900 μl of the standard saline (total volume 1 ml) with an anisotonic medium yielding the desired final osmolality. Recovery of pHᵢ from an acid load was determined using the NH₄Cl prepulse technique (8, 47). For this purpose, cells were exposed to 30 mM NH₄Cl for 5 min, followed by rapid washing with standard saline. At the end of each experiment, a three-point calibration was performed by replacing the experimental media with solutions of defined pH (pH 6.8, 7.2, 7.6) and high K⁺ (medium composition, in mM: 10 HEPES, 5.4 NaCl, 136.9 KCl, 1 MgSO₄, 0.33 NaH₂PO₄, 0.44 KH₂PO₄, 1.5 NaHCO₃, 1.5 CaCl₂, and 5 glucose) in the presence of the ionophores nigericin (10 μM) and valinomycin (5 μM).
presence of 7.2 μM of the calcium ionophore ionomycin, were determined to calibrate the measurements.

Statistics. Data are presented as means ± SE of the number (n) of independent preparations or individual cells. In the latter case, at least three cell cultures obtained from three different fish were used. Statistical differences were evaluated by applying analysis of variance (ANOVA) followed by the appropriate post tests. A value of \( P < 0.05 \) was considered significant.

RESULTS

Effect of cytoskeleton disrupting agents on F-actin and tubulin. Staining of the actin network in control hepatocytes revealed a mainly cortical localization of F-actin and a relatively even, low level of stained actin in the cytoplasm. Three-dimensional images reconstructed from 1-μm optical slices through the cells thus yielded a rather smooth, spherical appearance of the hepatocytes (Fig. 1A). Microtubular staining of control cells yielded a fine meshwork of tubules across the cytoplasm and clearly excluded the nuclear area (Fig. 1C). Images obtained by dual labeling of actin and tubulin showed a clear separation of both elements but indicated that at the cell periphery, microtubules may be in contact with cortical actin (Fig. 1, E–G). Incubation of trout hepatocytes with CB resulted in a disruption of actin filaments that was often visible as a disruption of the cortical actin ring and generally resulted in a punctate staining pattern of F-actin in three-dimensional projections of the cells (Fig. 1B). Qualitatively similar images were obtained from cells incubated with 2 μM CD, the CB analog, or with 2 μM latrunculin B (not shown). After exposure to colchicine for 30 min, microtubules still remained visible, but the associated fluorescence was diminished to a large extent (Fig. 1D).

Quantitative changes of F-actin distribution caused by CB and during anisotonic exposure. The distribution of F-actin was assessed by calculating the ratio between actin-fluores-
cence from the cortical and the cytoplasmic region of the cells. An example depicting a fluorescence profile obtained from an individual hepatocyte is shown in Fig. 2A. After exposure to 2 μM CB for 30 min, this method indicated a reduction of the cortical/cytoplasmic actin ratio to 78 ± 3% of that determined in control hepatocytes (Fig. 2B). During incubation with hypotonic medium, the cortical actin ring was reduced, both in thickness and in its fluorescence intensity (Fig. 2, E and G).

Fig. 2. Qualitative and quantitative assessment of cellular F-actin distribution in trout hepatocytes under control conditions, after treatment with CB, and after exposure to hypo- or hypertonic saline. A: image showing an optical section through the equatorial plane of a control cell (top) and the respective fluorescence profile used to quantify the distribution of cortical actin relative to central actin (bottom). B: normalized ratio of cortical actin fluorescence to that of central actin for hepatocytes under control conditions (isotonic saline, 284 mosM), after 30 min of exposure to CB, after 5 and 30 min of incubation in hypotonic saline (HYPO 5 and HYPO 30, 166 mosM), and after 5 and 30 min of incubation in hypertonic saline (HYPER 5 and HYPER 30, 465 mosM). Data are expressed as percentages of the mean of the control and are presented as means ± SE of 30 cells for each condition. *P < 0.05 compared with controls. C–H: representative images of cells subjected to the indicated treatments and subsequently stained for F-actin. Cont, control.
Changes were detected in hypertonic saline (Fig. 2, F and H), where the cortical/cytoplasmic actin ratio of hepatocytes remained constant at ~97% of the control value.

Changes of relative cell water volume. Changes of $V_r$ in response to hypo- and hypertonic exposure of hepatocytes are depicted in Fig. 3; a summary of the parameters used to characterize these alterations is given in Table 1. In control hepatocytes, hypotonic exposure caused an increase of $V_r$ to a maximum of $1.67 \pm 0.02$ after ~3 min (time 73 min in Fig. 3A), followed by a regulatory volume decrease (RVD) response of 53 ± 3 and 63 ± 4% after 20 and 40 min, respectively. Addition of CB to the cells did not affect $V_r$ under isotonic conditions or $V_{r\text{max}}$ attained in hypotonic saline, which amounted to 1.66 ± 0.03 in these cells. However, CB exposure affected subsequent cell volume recovery of the hepatocytes, which showed a tendency to be reduced after 20 min and was diminished by 33% ($P < 0.05$) after 40 min. In hypertonic saline, controls shrunk to a $V_{r\text{min}}$ of 0.62 ± 0.02 after 2 min (time 72 min in Fig. 3B) and showed a regulatory volume increase (RVI) of 39 ± 6 and 73 ± 6% after 20 and 40 min, respectively. Under these conditions, CB affected both $V_{r\text{min}}$, which amounted to 0.71 ± 0.02 ($P < 0.05$, as well as subsequent RVI, with all parameters determined being significantly reduced compared with untreated controls (Table 1).

Another series of experiments examined the impact of F-actin disruption on volume regulation in suspended hepatocytes to clarify whether cell attachment would be a prerequisite for the observed effects to occur. As shown in Fig. 4 and Table 1, the impact of CD on the regulatory volume changes of cells in suspension was qualitatively very similar to the effect exerted by CB on attached, cultured hepatocytes. Although in hypotonic saline, maximal cell swelling ($V_r = 1.29 \pm 0.03$ and 1.30 ± 0.16 in controls and CD-treated cells, respectively) was reduced in suspended hepatocytes compared with attached cells, CD nevertheless markedly inhibited RVD (Fig. 4A). Similarly, CD significantly reduced hypertonic RVI of the suspended hepatocytes (Fig. 4B). Interestingly, maximal cell shrinkage was identical in controls (0.80 ± 0.04, n = 10) and CD-exposed cells (0.80 ± 0.04, n = 7), ruling out the possibility that the slower rate of RVI of the latter was due to a smaller stimulus for volume recovery.

Table 1. Effect of CB (cell cultures), CD (cell suspensions), and cariporide on regulatory volume changes of hepatocytes exposed to hypo- or hypertonic saline

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<td></td>
<td>Controls</td>
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<td><strong>Cell cultures</strong></td>
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<tr>
<td>VRVC</td>
<td>3.70 ± 0.31</td>
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<tr>
<td>RVC20</td>
<td>53.2 ± 3.6</td>
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<tr>
<td>RVC60</td>
<td>62.8 ± 3.9</td>
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<tr>
<td><strong>Cell suspensions</strong></td>
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<tr>
<td>VRVC</td>
<td>11.66 ± 4.12</td>
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<tr>
<td>RVC20</td>
<td>74.9 ± 4.8</td>
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<tr>
<td>RVC60</td>
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Cells were exposed to conditions as described in the legends to Figs. 3 and 4. VRVC describes the initial rate of volume recovery in % per min, calculated by fitting data of regulatory volume change (RVC, %) vs. time (min) to the second-order polynomial $A_0 + V_{r\text{max}} t + C t^2$. RVC20 and RVC60 denote the extent of regulatory volume changes in % observed 20 min and 40 min after the maximum volume change, respectively. Data are means ± SE of the number of cells and independent experiments given in the legends to Figs. 3 and 4. *$P < 0.05$, significant differences compared with control cells. CB, cytochalasin B; CD, cytochalasin D.
after 30 min of incubation (Fig. 5B). A subsequent switch to hypertonic medium decreased the proton release rate by 28%, which was not significantly different from the decrease observed in controls. Exposure to hypertonic medium evoked an increase to 237% of the basal rate in controls, followed by a reduction to 159%, at which level it stabilized after ~15 min of incubation (Fig. 5C). In CB-treated cells, which again displayed the transient increase of proton release noted above, hypertonicity elevated acid secretion to 229% of the basal rate (P > 0.05 compared with control cells). However, in contrast to untreated hepatocytes, the acidification rate did not stabilize at an elevated level but returned to the basal level observed before the hypertonic challenge after 18 min (Fig. 5D). In cells incubated with CD, a nearly identical response was observed, with an immediate acid secretion maximum of 271% following a switch to hypertonic medium and a subsequent rapid recovery to the basal level within 18 min.

On the basis of our previous observation that Na\(^+\)/H\(^+\) exchange is strongly activated in trout hepatocytes during hypertonic exposure (37), we investigated how inhibition of this transporter would affect the acid release pattern observed in these conditions. As shown in Fig. 6, addition of the Na\(^+\)/H\(^+\) exchange inhibitor amiloride reduced acid secretion to 76 ± 4% of the initial value. More importantly, we observed that this treatment completely abolished the increase of proton release induced by hypertonic saline seen in the absence of the inhibitor.

In a separate series of experiments, we investigated the effect of the microtubule-disrupting agent colchicine. We observed that neither isotonic nor anisotonic rates of proton release were to any appreciable extent affected by this agent (Fig. 7).

**Intracellular pH**. As shown in Fig. 8A, in untreated hepatocytes, hypotonicity induced a decrease of pH\(_i\) by 0.15 units from a value of 7.36 ± 0.03 at time 0 to 7.21 ± 0.02 30 min later. In principle, CB-treated cells followed a similar pattern of hypotonic acidification, but in this case the initial and final values of pH\(_i\) were 7.18 ± 0.02 and 7.09 ± 0.02, respectively, and the change of pH\(_i\) (ΔpH\(_i\)) amounted to 0.09 units. Because hepatocytes had different starting pH\(_i\) values under both treatments, we converted ΔpH\(_i\) to net H\(^+\) accumulated, neglecting intracellular buffering capacity. This calculation yielded 12 ± 2 and 14 ± 2 nmol of H\(^+\) for controls and CB-treated cells, respectively (P > 0.05). As evaluated separately, the lower initial pH\(_i\) was due to CB, which caused a slowly progressing intracellular acidification in isotonic saline (Fig. 8A, inset).

In hypertonic medium, pH\(_i\) of control cells increased from a starting value of 7.39 ± 0.02 to a peak value of 7.76 ± 0.03 after 13 min, with ΔpH\(_i\) amounting to 0.37 units (Fig. 8B). The alkalization started immediately after the hypertonic shock and slightly declined again after the maximum. In contrast, in CB-treated cells (pH\(_i\) at time 0 = 7.24 ± 0.03), pH\(_i\) showed a slight initial decrease after the switch to hypertonic medium and subsequently displayed a biphasic response, with a more rapid increase in the first 10 min and a slower alkaline drift during the next 20 min (pH\(_i\) at 30 min = 7.42 ± 0.04). The ΔpH\(_i\) calculated for the entire period amounted to 0.17 units. After preincubation with latrunculin, cells had a starting pH\(_i\) of 7.32 ± 0.02 and reached a maximum pH\(_i\) of 7.54 ± 0.03 after ~10 min, corresponding to a ΔpH\(_i\) of 0.22 units. Converting ΔpH\(_i\) values to the net amount of H\(^+\) extruded, we obtained...
24 ± 2, 20 ± 4, and 19 ± 2 nmol H+ in controls, CD-treated cells, and latrunculin-treated cells, respectively (P > 0.05).

Prompted by our findings on proton secretion rates, we suspected that these different pHi response patterns could have been caused by an effect of cytoskeletal disruption on the Na+/H+ exchanger. To investigate this possibility, we examined the pHi recovery in controls and CB-treated hepatocytes after the induction of an acid load, a condition known to strongly activate this ion transporter in trout hepatocytes (23, 60, 63). Intracellular acidification was achieved using the NH4+ prepulse technique (8, 47) with concurrent addition of the inhibitor of Na+-dependent Cl-/HCO3− exchange, SITS, to prevent compensatory activation of this transporter should Na+/H+ exchange indeed have been diminished by the CB treatment. As depicted in Fig. 9, addition of NH4+ resulted in a pronounced alkalinization and, after the washout of extracellular NH4+ (pHi 0.25 compared with the resting value). In controls, this was followed by gradual recovery of pHi toward the resting level. In comparison, in CB-treated hepatocytes, a slightly but not significantly larger increase of pHi was induced by addition of NH4+. However, a much less pronounced acidification was noted upon removal of extracellular NH4+ (pHi 0.09), and there was no pHi recovery to be noted subsequently.

Similar to our findings on acid secretion rates, preincubation of hepatocytes with colchicine had no significant effects on pHi under isotonic conditions and also did not alter pHi changes evoked by anisotonic conditions (data not shown).

Cytosolic free Na+ concentration. To validate our method employed for the determination of [Na+]i, we initially exposed hepatocytes to the Na+-K+-ATPase inhibitor ouabain. As to be expected, this caused Na+ to slowly accumulate in the cells, starting from a basal level for [Na+]i of 19 mM to a value of 44 mM after 30 min of incubation with ouabain (Fig. 10, inset). In isotonic saline, we noted a slight increase of [Na+]i in controls from 16 ± 1 mM at time 0 to 19 ± 1 mM after 30 min. During incubation with CB, this increase was slightly but significantly more pronounced, elevating [Na+]i from 17 ± 1 mM to 22 ± 1 mM within this period. The impact of hypertonic conditions on [Na+]i is depicted in Fig. 10. In control cells, hypertonicity caused an immediate increase of [Na+]i, to 24 ± 1 mM within the first minute of exposure to a peak at 32 ± 1 mM after ~8 min and a slight subsequent decrease to 30 ± 1 mM after 30 min. In hepatocytes pretreated with CB, the initial increase of [Na+]i was significantly larger and reached 29 ± 1 mM after 1 min, followed by a further gradual increase to a final value of 47 ± 1 mM at the end of the 30-min period of investigation.
As shown below, CB significantly diminished Na\(^+\)-K\(^+\)-ATPase activity. Therefore, we tested whether the elevated increase of [Na\(^+\)]\(_i\) in CB-treated cells could have been a result of this inhibition. As shown in Fig. 10, [Na\(^+\)]\(_i\) of ouabain-treated cells increased to 20 ± 2 mM and further to 27 ± 2 mM after 1 and 2 min of hypertonic exposure, respectively. Thereafter, [Na\(^+\)]\(_i\) indeed showed an increase similar to that noted in CB-treated cells, with [Na\(^+\)]\(_i\) reaching a final value of 52 ± 3 mM at 30 min. We also examined the possibility that reduced Na\(^+\)/K\(^+\) exchanger activity could underlie the elevated [Na\(^+\)]\(_i\). However, the presence of cariporide did not alter the increase of Na\(^+\), with [Na\(^+\)]\(_i\) reaching a value of 23 ± 1 after 1 min, a first plateau at 31 ± 1 mM after 5 min, and a final value of 35 ± 2 mM after 30 min (n = 51 cells from 3 independent preparations).

Na\(^+\)-K\(^+\)-ATPase activity. Another series of experiments addressed the impact of cytoskeletal disruption on both the primary active uptake of K\(^+\) via Na\(^+\)-K\(^+\)-ATPase activity as well as on ouabain-resistant uptake of K\(^+\). In control cells, the rates of total and ouabain-resistant Rb\(^+\) uptake amounted to 0.53 ± 0.05 and 0.04 ± 0.03 nmol·10⁶ cells\(^{-1}\)·min\(^{-1}\) (n = 6), respectively, and the difference, i.e., Rb\(^+\) uptake mediated by Na\(^+\)-K\(^+\)-ATPase, amounted to 0.48 ± 0.03 nmol·10⁶ cells\(^{-1}\)·min\(^{-1}\). In comparison, after 30 min of incubation with CB, total Rb\(^+\) uptake was significantly reduced to 0.25 ± 0.08 nmol·10⁶ cells\(^{-1}\)·min\(^{-1}\) (n = 5), whereas ouabain-resistant uptake was slightly increased to 0.11 ± 0.05 nmol·10⁶ cells\(^{-1}\)·min\(^{-1}\) (P > 0.05). The active rate of Rb\(^+\) uptake therefore amounted to 0.14 ± 0.13 nmol·10⁶ cells\(^{-1}\)·min\(^{-1}\), which corresponds to a 71% reduction compared with the controls.

Cytosolic free Ca\(^{2+}\) concentration. Finally, we investigated the effect of CB on changes of [Ca\(^{2+}\)]\(_i\), elicited by anisotonic challenges, an important element in the volume regulatory response of various cells. In line with previous observations (37), we found that hypotonic exposure evoked only a slight, gradually developing elevation of [Ca\(^{2+}\)]\(_i\) in trout hepatocytes, and neither the pattern nor the extent of this increase was
significantly affected by CB (data not shown). In contrast, in hypertonic saline, control hepatocytes showed a rapid increase of \([\text{Ca}^{2+}]_i\) from an initial level of 92 ± 11 nM to a peak of 160 ± 20 nM after 6 min, where \([\text{Ca}^{2+}]_i\) was maintained with some fluctuations during the rest of the experimental period (Fig. 11). In CB-treated cells, baseline \([\text{Ca}^{2+}]_i\) was 57 ± 6 nM at time 0 and increased to 89 ± 9 nM upon hypertonic exposure, with this elevation being significantly lower than in controls. Thereafter, this elevated value remained relatively constant.

**DISCUSSION**

The present study provides various lines of evidence that the actin-based cytoskeleton plays an important, multifaceted role for cell volume regulation of trout hepatocytes. We found that the distribution of actin filaments remained unaffected under hypertonic conditions but was significantly altered during hypotonic exposure of the cells. Within 5 min of hypotonic incubation, cortical actin was significantly reduced and showed only a slight tendency for recovery during 30 min. Quantitatively, these changes corresponded to a 15–20% reduction in the ratio of cortical to central F-actin fluorescence (\(P < 0.05\)) during this period. This relative decrease of cortical F-actin agrees with similar observations on various other cells (26, 54), including chondrocytes, where the same method was used (17).
The lack of an effect of hypertonicity on F-actin distribution also has been reported for some other cells (17) but is at variance with some studies in which an increase of F-actin has been observed (25, 54, 57). These results clearly demonstrate that the actin-based cytoskeleton is affected by cell volume changes in many cells but that there may be cell type-specific differences with respect to the exact mechanism of this modification (53).

In addition to the hypotonic effect on the actin-based cytoskeleton, we observed that disruption of the F-actin by use of cytochalasins affected both cell volume changes elicited by anisotonic challenges and ionic fluxes associated with volume regulation in trout hepatocytes. Several previous studies failed to document such effects, but at least in some cases this appears to be due to technical difficulties. For example, the concentration of CB or one of its analogs adequate to disrupt F-actin in one cell type may be inadequate to disrupt the F-actin in another cell line (15). Furthermore, cytochalasins may even increase, instead of decrease, F-actin when used at high concentrations (21). To exclude this possibility, we both qualitatively and quantitatively assessed the impact of CB on F-actin of trout hepatocytes. As shown in Figs. 1 and 2, the mainly cortical localization of F-actin in control hepatocytes, a feature typical for many cells, was clearly disrupted by CB at the concentration and the preincubation period applied. As a result of this, the cortical/cyttoplasmic actin ratio was diminished by −20% compared with the control value.

Although quantitatively this decrease corresponded to that observed in hypotonically exposed cells, its physiological effect was quite dissimilar, leading to an inhibition of RVD. The actin rearrangement induced by cell swelling therefore seems to be a more subtle and controlled process, which may either not affect or possibly even enhance volume regulatory responses. In contrast, the rather nonspecific disruption of F-actin by CB appears to destroy structures required for normal cell volume regulation and therefore does not mimic the hypotonic alterations. In line with this finding, F-actin disruption significantly reduced RVI of hepatocytes, although we could not detect a change of F-actin distribution under hypertonic conditions. Thus the integrity of F-actin may not directly control, but could act as a permissive factor for, the function of transporters involved in volume regulatory processes.

In agreement with this notion, neither maximum swelling nor the initial rate of volume recovery was affected by CB or CD, which is consistent with previous reports (15, 19, 54). Subsequently, however, CB and CD delayed volume recovery, suggesting a possible link between actin filaments and volume regulatory transporters, e.g., K+/Cl− exit pathways, which became activated upon swelling in many cells (30, 41), including trout hepatocytes (6, 52). An interaction between F-actin and these transporters has been previously documented for mammalian cells (9, 33, 50). In the present study, we concentrated on proton-linked ion transport pathways, and therefore we can only speculate on the possible connection between the actin-based cytoskeleton and K+/Cl− exit pathways that do not involve the net transport of acidic equivalents. Although disruption of F-actin significantly attenuated the RVD response of hepatocytes compared with control cells, the decrease in the rate of acid secretion during hypotonic exposure was not affected by CB. This finding clearly indicated that CB inhibited ion release of hepatocytes independent of proton release, and this inhibition impaired the RVD response. Accordingly, the intracellular acidification of CB-treated hepatocytes following hypotonic exposure was not altered compared with control cells, yielding similar amounts of H+ accumulated over 30 min. The relatively minor role of acid-transporting pathways in RVD is also underscored by the lack of any effect of Na+/H+ exchange inhibition on volume recovery. Although this may be typical for many cells, activation of Na+/H+ exchange upon swelling has in fact been observed in certain cell types (46) and under certain conditions (51).

In contrast to hypotonic conditions, during hypertonic exposure, CB treatment reduced cell shrinkage in attached cells compared with controls but did not alter that of cells in suspension. The reasons underlying this discrepancy remain unknown at present. However, our data show that in both cell cultures and suspended hepatocytes, a significantly diminished extent of volume recovery after 20 and 40 min can be observed. This finding suggests that in neither hypotonic nor hypertonic conditions is cell attachment prerequisite for an effect of F-actin disruption on volume regulatory processes. In the latter case, our study provides direct evidence that this is due to an interaction of the actin-based cytoskeleton and the activity of ion transporters. As an outstanding candidate, we identified the Na+/H+ exchanger, because its inhibition by cariporide reduced RVI by −60%. In control cells, hypertonic saline evoked a pronounced increase of proton secretion, the time course and pattern of which largely paralleled the changes in cell volume. In addition, these changes were accompanied by a substantial alkalinization, which, as previously shown (20, 37), is entirely due to enhanced activity of the Na+/H+ exchanger. This was further corroborated by showing that in the presence of amiloride, hypertonic exposure did not result in an increase of proton release. Recent experiments in which the rather specific NHE-1 inhibitor cariporide was used confirmed these findings. Cariporide completely inhibited the hypertonic alkalinization, and it also blocked most, but not all, of the hypertonicity-induced increase of proton release. This finding suggests that other transporters mediating net acid secretion were concurrently activated in hypertonic saline and that the complete inhibition seen with amiloride was in part due to nonspecific effects of the drug on these transporters (Ahmed K, Pelster B, and KrumSchnabel G, unpublished observation).

In CB-treated cells, the hypertonicity-induced increase of proton release was only short-lived and pHt was transiently even decreased, followed by a significantly delayed elevation of pHt compared with the controls. In comparison, latrunculin did not acidify hypertonically exposed cells, and it reduced the subsequent increase of pHt. Expressed as H+ extruded, the reduction in the intracellular alkalinization corresponded to a decrease in proton secretion by −20%. Thus we conclude that the activity of the Na+/H+ exchanger is, at least in part, under the control of the actin-based cytoskeleton, with an intact cytoskeleton required for full activation of this transporter under hypertonic conditions. In principle, this finding is in line with other reports, although the best documented example in this regard is the NHE-3 isoform of the Na+/H+ exchanger family (40, 58), which is not involved in the RVI response, because it is inhibited, rather than activated, by osmotic shrinkage (34). However, a close link between NHE-1, the housekeeping isoform related to regulation of cell volume and pHt in most cells (62), and the cytoskeleton has recently been firmly
established (14, 56), and an inhibition of hypertonically induced alkalization, relying on Na\(^{+}/H^{+}\) exchanger activity, by CB has been previously described for an osteoblast-like cell line (13).

To further substantiate our conclusion on the dependence of Na\(^{+}/H^{+}\) exchange activity on an intact actin-based cytoskeleton, we also investigated the impact of CB on pH\(_{i}\) recovery following an acid load, which, as shown in previous studies on trout hepatocytes (23, 60, 63), is to a major extent dependent on Na\(^{+}/H^{+}\) exchange and, to a minor extent, on a SITS-sensitive anion transporter. We observed that upon acidification after an NH\(_{4}\)\(^{+}\) prepulse in the presence of SITS, control cells clearly recovered pH\(_{i}\), whereas in CB-treated cells, no such recovery was noted. For yet unknown reasons, the initial acidification was less pronounced in the latter group, but as shown by Walsh (63), even such a minor decrease of pH\(_{i}\) should be sufficient to induce a pH\(_{i}\) regulatory response. Finally, the decrease of pH\(_{i}\) induced by CB in isotonic saline may be taken as another argument in favor of a partial inhibition of Na\(^{+}/H^{+}\) exchange in cells with a disrupted cytoskeleton, because 1) this transporter is known to be tonically active, as indicated by the reduction of proton release in the presence of amiloride (present study and Ref. 37), and 2) inhibition of the exchanger leads to a similar decrease of steady-state pH\(_{i}\) (22, 23).

Na\(^{+}/H^{+}\) exchange transports Na\(^{+}\) into the cell and thus obviously is of osmoregulatory and volume regulatory importance. However, in rat hepatocytes, when exposed to a comparable hypertonic challenge, its contribution to net Na\(^{+}\) uptake was estimated to be ~15%, whereas the main route of Na\(^{+}\) entry was via conductive pathways (65). In line with this, we observed that in the presence of cariporide, changes of [Na\(^{+}\)] \(_{i}\) during hypertonic exposure were unaltered compared with controls. Similarly, in CB-treated trout hepatocytes, despite apparently inhibited Na\(^{+}/H^{+}\) exchange, the increase of [Na\(^{+}\)] \(_{i}\) was not inhibited but augmented during hypertonic exposure. On the basis of previous observations (3, 10), this could be tentatively linked to a stimulatory effect on Na\(^{+}\)\(^{+}\) exchange activity of short actin filaments produced by the net inhibitory effect of CB on F-actin assembly. Alternatively, the maintenance of low ion permeability by actin-binding proteins such as filamin (10) may have been disturbed by the disruption of the actin cytoskeleton. Our data also indicate that a modification of Na\(^{+}\)-K\(^{+}\)-ATPase activity in CB-treated trout hepatocytes may have contributed to the elevation of [Na\(^{+}\)] \(_{i}\). After hypertonic exposure, Na\(^{+}\)-K\(^{+}\)-ATPase is known to be tonically active, as indicated by the reduction of proton release in the presence of amiloride (present study and Ref. 37), and 2) inhibition of the exchanger leads to a similar decrease of steady-state pH\(_{i}\) (22, 23).

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osmotically relevant ion transport mechanisms that do not transport acid or base equivalents in parallel.

Besides these direct effects of Na\(^{+}/H^{+}\) exchange on osmotic and cell volume regulation, it should be pointed out that an inhibition of this transporter may elicit indirect effects. Efflux pathways for K\(^{+}\) and Cl\(^{-}\) involved in RVD have been found to be inhibited at low pH (31), which could in part account for the slowed RVD of cytochalasin-treated cells. On the other hand, Na\(^{+}/H^{+}\) exchange activity is stimulated at low pH, and thus the decreased pH\(_{i}\) should accelerate RVI and not inhibit it. To clarify this matter, investigation of the pH dependence of volume recovery in trout hepatocytes is currently under way.

A final aspect we addressed in the present study was the potential impact of cytoskeletal disruption on [Ca\(^{2+}\)] \(_{i}\), changes evoked by anisotonic exposure. Although the importance of Ca\(^{2+}\) \(_{i}\) in volume regulation is not fully understood, several studies indicate an important role, and more importantly, there is evidence for functional interactions between intracellular Ca\(^{2+}\) and the cytoskeleton (7, 32). With regard to the latter, Erickson et al. (17) recently showed that, in chondrocytes, F-actin redistribution evoked by hypotonic exposure was strictly dependent on [Ca\(^{2+}\)] \(_{i}\), oscillations induced in the presence of extracellular Ca\(^{2+}\). Although this was not tested in the present study, we saw that hypotonicity induced an increase of [Ca\(^{2+}\)] \(_{i}\), and a redistribution of F-actin but that disruption of the cytoskeleton had no effect on [Ca\(^{2+}\)] \(_{i}\), changes. At the same time, however, the CB treatment significantly diminished the [Ca\(^{2+}\)] \(_{i}\), increase elicited in hypertonic saline. The importance of intracellular Ca\(^{2+}\) \(_{i}\) in the RVI response of trout hepatocytes is not known, but at least the NHE-1 isoform of Na\(^{+}/H^{+}\) exchangers is known to be stimulated by an increase of [Ca\(^{2+}\)] \(_{i}\), via a cytoplasmic calmodulin binding site, the deletion of which drastically inhibits its hypertonicity-induced activation (4, 61).

Of further note is that none of the parameters investigated appeared to be in any way significantly affected by colchicine incubation, despite the fact that our visual inspection indicated that the microtubular meshwork was indeed disrupted by this treatment. Therefore, in agreement with other studies (11, 12), we conclude that intact microtubules are not a prerequisite for normal cell volume regulation, at least not during short-term anisotonic exposure.

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REFERENCES


