Hyperosmotic stress activates Rho: differential involvement in Rho kinase-dependent MLC phosphorylation and NKCC activation

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First published May 14, 2003; 10.1152/ajpcell.00086.2003.—Hyperosmotic stress initiates adaptive responses, including phosphorylation of myosin light chain (MLC) and concomitant activation of Na+/K+–Cl− cotransporter (NKCC). Because the small GTPase Rho is a key regulator of MLC responses that can be classified into three major categories: activation of ion transport systems; changes in the transcription of osmosensitive genes and, consequently, in the expression of osmolyte-generating enzymes or solute carriers; and reorganization of the cytoskeleton (for reviews, see Refs. 28, 35, 38, and 53). Whereas considerable knowledge has accumulated about these vital adaptive responses, comparatively little is known about the signaling processes that link them to the initial osmotic insult. Regarding the cytoskeletal effects, our previous studies (8, 29) have shown that Rac and Cdc42, two members of the Rho family small GTPases, are stimulated by hyperosmotic stress and that their activation contributes to the osmotically provoked cytoskeleton remodeling. Specifically, these small GTPases appear to play a role in the reinforcement of the cortical cytoskeleton by facilitating peripheral de novo F-actin assembly (8, 29) and by inducing translocation of cortactin (8), a protein that potentiates actin polymerization and stabilizes newly generated actin networks (51, 52).

In addition to F-actin remodeling, another prominent cytoskeletal response to hyperosmotic stress is the phosphorylation and/or redistribution of myosin. Increased phosphorylation of the regulatory myosin light chain (MLC) has been documented in hyperosmotically treated mesangial cells (46), endothelial cells (23, 34), and astrocytes (42), and peripheral myosin translocation was recently reported in Ehrlich ascites cells (37). Moreover, myosin phosphorylation and redistribution has been shown to be essential for survival after cell shrinkage in Dictyostelium (25, 54). Functionally, the myosin response may not only serve to increase cortical rigidity, and thereby resistance against osmotic stress, but also has been implicated in the volume-dependent regulation of the Na+/K+–Cl− cotransporter (NKCC) (23, 34), a key player in cell volume homeostasis (13). This assumption has been based on pharmacological observations showing that ML-7, a myosin light chain kinase (MLCK) inhibitor, abrogated the hypertonic stimulation of NKCC (23, 34). However, neither the mechanism responsible for osmotic MLC phosphorylation nor the relationship between MLC phosphorylation and NKCC activation has been conclusively elucidated.

OSMOTIC STRESS induces a variety of compensatory responses that can be classified into three major categories: activation of ion transport systems; changes in the transcription of osmosensitive genes and, consequently, in the expression of osmolyte-generating enzymes or solute carriers; and reorganization of the cytoskeleton (for reviews, see Refs. 28, 35, 38, and 53). Whereas considerable knowledge has accumulated about these vital adaptive responses, comparatively little is known about the signaling processes that link them to the initial osmotic insult. Regarding the cytoskeletal effects, our previous studies (8, 29) have shown that Rac and Cdc42, two members of the Rho family small GTPases, are stimulated by hyperosmotic stress and that their activation contributes to the osmotically provoked cytoskeleton remodeling. Specifically, these small GTPases appear to play a role in the reinforcement of the cortical cytoskeleton by facilitating peripheral de novo F-actin assembly (8, 29) and by inducing translocation of cortactin (8), a protein that potentiates actin polymerization and stabilizes newly generated actin networks (51, 52).

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phosphorylation and NKCC activation have been fully elucidated.

One of the major upstream regulators of MLC phosphorylation is Rho. Active (GTP bound) Rho stimulates Rho kinase (ROK), which in turn can increase MLC phosphorylation by two distinct mechanisms: first, it can catalyze direct mono- and diphosphorylation of MLC (48, 50); and second, it phosphorylates, and thereby inactivates, myosin light chain phosphatase (MLCPase) (21), an effect that shifts the balance toward elevated MLC phosphorylation.

Our previous findings that other Rho family members are osmotically sensitive (8, 29), together with the fact that MLC is phosphorylated upon hypertonic stress (23, 42, 46), provide a strong rationale for our hypothesis that Rho GTPase itself might be regulated by hypertonic stress. Interestingly, recent studies have implicated intact Rho signaling (presumably as a requirement for cytoskeletal integrity) in the hyposmotic shock-induced ionic responses (11, 25, 36). However, hypotonic stress was found not to stimulate Rho (7), suggesting that activation of Rho is not necessary and that Rho plays a permissive role in the hypertonicity-induced responses (7).

Given this scenario, the central aim of the present study was to discern whether Rho plays an active role in the mediation of hypertonic responses. Specifically, we wanted to establish whether Rho is stimulated by hyposmotic stress and, if so, whether the Rho/ROK pathway contributes to the hyposmotically induced MLC phosphorylation. We also wanted to examine which physical or chemical changes (cell volume, ionic concentrations, ionic strength) may trigger Rho activation in hyposmotically challenged cells. Finally, we addressed whether ROK may be involved in the hypertonic stimulation of NKCC and revisited the intriguing question of whether there is a causal coupling between the hypertonic MLC phosphorylation and NKCC activation. Our results show that hyposmotic stress induces a rapid and robust activation of Rho in kidney proximal tubule cells. Both cell shrinkage and elevated ionic strength are capable of eliciting Rho activation, and the Rho/ROK pathway contributes to the basal and osmotic MLC phosphorylation. On the other hand, ROK activation is not necessary for the osmotic stimulation of NKCC, and thus a substantial portion of MLC phosphorylation can be uncoupled from NKCC activation.

MATERIALS AND METHODS

Reagents and Antibodies

Y-27632 and ML-7 were from Calbiochem. Nystatin, ouabain, and bumetanide were from Sigma. Toxin B isolated from Clostridium difficile (ToxB) was purchased from TechLab, and calyculin A was from Biomol. Protease inhibitor cocktail (0.8 mg/ml benzamidine hydrochloride, 0.5 mg/ml aprotinin, 0.5 mg/ml leupeptin, 0.5 mg/ml pepstatin A, and 50 mM PMPS) in pure ethanol was obtained from Pharmingen. Glutathione-Sepharose beads were from Amersham Pharmacia Biotech. Monoclonal anti-RhoA/B/C antibody, polyclonal anti-MLC antibodies, and peroxidase-conjugated anti-goat IgG were from Santa Cruz Biotechnology. Peroxidase-conjugated anti-mouse and anti-rabbit IgG were from Amersham Pharmacia Biotech. A small aliquot of polyclonal phospho-Ser19-specific MLC antibody was a kind gift from Dr. Matsumura (Rutgers University, New Brunswick, NJ). All other reagents were of the highest purity commercially available.

Cell Culture

Kidney tubular epithelial LLC-PK1 cells were grown under a humidified atmosphere of air-CO\(_2\) (19:1) at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotic suspension (penicillin and streptomycin; Sigma) as described previously (31).

Media

For serum depletion, bicarbonate-free RPMI 1640 was buffered with 25 mM HEPES to pH 7.4 (HPMI, 290 \pm 5 mOsm). The following media were used for treatments. The isotonic sodium chloride medium (Iso-NaCl) consisted of (in mM) 130 NaCl, 3 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 20 HEPES (pH 7.4), and 5 glucose. For \(^86\)Rb uptake experiments, Iso-NaCl was supplemented with 2 mM extra KCl (final 5 mM) and 0.3 mM Na\(_2\)HPO\(_4\). The composition of the isotonic sodium nitrate solution (Iso-NaNO\(_3\)) was identical to that of Iso-NaCl, except all chloride salts were replaced with the corresponding nitrates. The isotonic N-methyl-D-glucammonium aspartate medium (Iso-NMDG) consisted of (in mM) 140 NMDG, 140 aspartic acid, 3 NaCl, 1 MgCl\(_2\), 1 EGTA, 0.194 CaCl\(_2\), 10 HEPES (pH 7.1), and 5 glucose. The intracellular-like potassium chloride medium (Intra-KCl) consisted of (in mM) 140 KCl, 10 NaCl, 1 MgCl\(_2\), 1 EGTA, 0.194 CaCl\(_2\), free [Ca\(^{2+}\)] 100 nM, 10 HEPES (pH 7.2), 5 glucose, and 60 sucrose (to counterbalance the colloid osmotic pressure). To permeabilize cells for monovalent ions, the Intra-KCl solution was supplemented with 400 U/ml nystatin as described previously (44). The various media were made hyposmotic by the addition of excess sucrose, NaCl, KCl, or NaNO\(_3\), as specified.

Cell Treatment

For all experiments, LLC-PK1 cells were grown to confluence, serum-deprived in HPMI for 3 h, and then preincubated with Iso-NaCl solution for 10–20 min. They were then treated as indicated.

Preparation of Glutathione-S-Transferase-Rho-Binding Domain Fusion Protein

Escherichia coli were transformed with a construct encoding for a glutathione-S-transferase (GST) fusion protein containing the RhoA-binding domain (RBD; amino acids 7–89) of Rhotecin (GST-RBD). GST-RBD was then purified as described previously (4) with a small modification. Briefly, transformed E. coli were induced with 0.4 mM isopropyl thioglycolic acid (3 h), sedimented, and lysed in ice-cold STE buffer [150 mM NaCl, 10 mM Tris (pH 8.0), and 1 mM EDTA] supplemented with 5 mM DTT, 100 \mu g/ml lysozyme, 20 \mu g/ml protease inhibitor cocktail, and 1 mM PMPS. Lysates were subjected to a pressure of 900 lb./in.\(^2\), followed by the addition of 1% sarcosyl and centrifugation at 12,000 rpm for 10 min (4°C). The supernatant was supplemented with 1% Triton X-100 and incubated with glutathione-Sepharose beads for 1 h. The GST-RBD-covered beads were washed extensively with STE buffer and stored at 4°C.
**Rho Activity Assay**

LLC-PK1 cells grown in 10-cm dishes were treated as described and lysed in 800 μl of ice-cold lysis buffer [100 mM NaCl, 50 mM Tris-base (pH 7.6), 20 mM NaF, 10 mM MgCl\textsubscript{2}, and 1% Triton X-100] supplemented with 0.5% deoxycholic acid, 0.1% SDS, 20 μg/ml protease inhibitor cocktail, 1 mM Na\textsubscript{3}VO\textsubscript{4}, and 1 mM PMSF. Cells were immediately scraped from the dishes and briefly clarified by centrifugation at 12,000 rpm for 1 min at 4°C. Glutathione-Sepharose beads (10–15 μg) covered with GST-RBD were then added to the supernatants and incubated at 4°C for 45 min. The GST-RBD-covered beads were then washed extensively, and the captured proteins were diluted with 25 μl of Laemmli buffer and subjected to Western blotting. For positive and negative controls, LLC-PK1 cells were transfected with 4 μg of DNA encoding for Myc-tagged constitutively active (Q63L) or dominant negative (T19N) Rho (45), using FuGENE6 (Roche Molecular Biochemicals). Forty-eight hours later, the cells were lysed and active Rho was precipitated as described above.

**Western Blotting**

Protein samples diluted in Laemmli buffer were boiled for 5 min, subjected to SDS-PAGE on 15% gels, and transferred to nitrocellulose by using a semidy apparatus (Bio-Rad). Blots were blocked in Tris-buffered saline containing 5% bovine serum albumin and incubated with the corresponding primary antibody. Binding of the antibody was visualized by using the relevant (anti-mouse, -rabbit, or -goat) peroxidase-conjugated secondary antibodies (1:3,000 dilution) with the appropriate test solution (see Fig. 2 for details), and their incubation was followed by enhanced chemiluminescence method.

**MLC Phosphorylation**

**Sample preparation.** Non-, mono-, and diphosphorylated forms of MLC were separated using nondenaturing urea-glycerol PAGE (31). After treatment, LLC-PK1 cells grown in six-well plates were lysed in 850 μl of ice-cold acetone containing 10% TCA and 10 mM DTT, followed by centrifugation for 10 min at 12,500 rpm (4°C). The resulting pellet was washed with 1 ml of pure acetone, allowed to air dry, and dissolved in 0.2 ml of sample buffer containing 8.02 M urea, 234 mM sucrose, 23 mM glycerol, 10.4 mM DTT, 20 mM Tris (pH 8.6) and 0.01% bromphenol blue. After resolubilization, samples were centrifuged for 10 min at 12,500 rpm and the supernatants were loaded into urea-glycerol gels.

**Electrophoresis.** Urea-glycerol (10%) gels were prerun for 1 h at 200 V in running buffer containing 23 mM glycerol, 20 mM Tris (pH 8.6), 2.44 mM thioglycolic acid, and 0.97 mM DTT. The samples were then loaded and run at 280 V for 2.5–3.0 h. To quantify phosphorylation, the total amount of MLC was determined in each lane by densitometry, and the amount of mono- and diphosphorylated forms was expressed as a percentage of the total MLC in the same lane. This normalization allows direct comparison among the samples and prevents any potential errors that might arise from differences in loading.

**Densitometry**

Densitometric analysis of blots was performed using a Bio-Rad model GS-690 imaging densitometer and Molecular Analyst software (version 1.5) as described previously (19).

**Measurement of Cell Volume**

Cell volume was determined using electronic sizing, as in our previous studies (18). Confluent LLC-PK1 cells were trypsinized, sedimented, and washed three times with ISO-NaCl solution. Cells were then washed in 20 ml of the appropriate test solution (see Fig. 2 for details), and their volume was measured using a Coulter counter model ZM, equipped with a Channelyzer.

**Measurement of Cellular Cl\textsuperscript{−} Content**

After the appropriate preincubations, LLC-PK1 cells were washed thoroughly with ice-cold Iso-NaCl\textsubscript{3} medium, lysed with 1 mM nitric acid, and sedimented. The supernatant was collected and mixed with an equal volume of the photometric determination mixture (1 part mercuric thiocyanate in 0.417% methanol solution, 1 part 20.2% ferric nitrate, and 13 parts water) (1). The absorbance of the test samples and Cl standards was then determined at 480 nm by using a Beckman-Coulter DU 640 spectrophotometer.

**Determination of NKCC Activity**

NKCC activity was monitored as the bumetanide-sensitive uptake of 86Rb (3). Briefly, serum-depleted LLC-PK1 cells were incubated in Iso-NaCl for 20 min in the absence or presence of Y-27632 (20 μM) or ML-7 (75 μM). When present, these inhibitors were added to all subsequent solutions. Thereafter, the cells were incubated in Iso-NaCl containing ouabain (1 mM) for 2 min in the presence or absence of bumetanide (100 μM). They were then treated iso- or hypertonically for 7 min in the corresponding medium supplemented with 1 mM ouabain and 1.5 μCi of 86Rb, with or without bumetanide. Hypertonicity was achieved by the addition of 100 mM sucrose to the treatment media. At the end of the uptake period, the cells were quickly washed five times with ice-cold 0.1 M MgCl\textsubscript{2}, air-dried, and dissolved in 0.2% SDS. The amount of accumulated 86Rb was measured using a Beckman-Coulter LS-6500 scintillation counter. Three separate experiments were performed in which each condition was done in triplicate. Bumetanide-sensitive uptake is expressed in nanomoles of K\textsuperscript{+} per 7 min.

**Protein Assay**

Protein concentration was determined by bicinchoninic acid assay (BCA assay; Pierce) with BSA used as a standard.

**Statistical Analysis**

Data are presented as representative blots of at least three similar experiments or as the means ± SE of the number of experiments indicated (n). Statistical significance was assessed by ANOVA, using SAS software.

**RESULTS**

**Hyperosmotic Stress Activates the Small GTPase Rho**

To assess the effect of hyperosmolality on Rho activity, we compared the amount of active Rho present in lysates obtained from iso- or hypertonically treated kidney tubular (LLC-PK1) cells by using a recently developed pulldown assay. This approach relies on a fusion protein composed of GST and the Rho-binding domain of Rhotekin, a downstream effector of Rho, which binds the active (GTP bound) form but fails to react with the inactive (GDP bound) form of Rho (4). Cells were exposed to either isotonic (290 mosM) or hypertonic (590 mosM) media for 10 min, followed by lysis. Cleared lysates were then incubated with gluta-
thione beads covered with GST-RBD, and the amount of captured Rho protein was determined by Western blotting. Figure 1A shows that hypertonic treatment induced substantial Rho activation. When osmolarity was raised to the same level by 150 mM NaCl or 300 mM sucrose, the amount of active Rho increased 2.74 ± 0.33-fold (n = 12) and 3.00 ± 0.36-fold (n = 11) compared with the isotonic level, respectively. Thus ionic and nonionic osmolytes are equally capable of inducing Rho activation at comparable external osmolarities. Cell volume measurements made using electronic sizing verified that both osmolytes provoked cell shrinkage of similar magnitude (~50%), implying that under these conditions the cells behaved as nearly perfect osmometers and remained shrunken throughout the time course of the measurement (Fig. 2, columns 1–3). Next, we examined the kinetics of the Rho response. As shown in Fig. 1B, the osmotic stimulation was rapid and sustained: near-maximal elevation was attained after 1 min, reaching a plateau between 2 and 10 min at ~3.5-fold activation and slightly decaying by 30 min. Under isotonic conditions there was no detectable change in Rho activity in the investigated time range (Fig. 1B). Although osmotic stress increased Rho activity, it did not affect the total amount of Rho in the lysates (Fig. 1, A and B). To confirm the specificity of the GST-RBD beads and validate the observed changes, we performed control experiments. LLC-PK1 cells were transiently transfected with Myc-tagged dominant negative (DN) or constitutively active (CA)
versions of Rho. As expected, CA Rho but not DN Rho associated with GST-RBD (Fig. 1B).

To further characterize the Rho activation response, we investigated its osmotic dependence and reversibility. The level of Rho stimulation was proportional to the applied osmotic concentration (Fig. 1C). In addition, upon restoration of isotonicity, GTP-Rho levels rapidly (within a few minutes) decreased back to or even below prestimulus values (Fig. 1D). Taken together, these results show that hyperosmotic stress induces rapid, sustained, and reversible Rho activation that is proportional to the applied osmolarity.

**Increased Ionic Strength and Decreased Cell Volume Are Each Capable of Inducing Rho Activation**

Having shown that Rho is a sensitive indicator of hyperosmotic stress, we investigated which parameters trigger Rho activation in osmotically challenged cells. An increase in the extracellular concentration of impermeable osmolytes results in an instantaneous decrease in cell volume and a simultaneous increase in the intracellular solute concentrations and ionic strength. To delineate the role of these factors, we applied experimental conditions, which allow their selective manipulation. First we addressed whether an elevation in intracellular Na\(^+\) concentration itself may be sufficient to induce a robust rise in Rho activity.

Although increased ionic strength was unambiguously capable of activating Rho, the above experiments did not exclude the possibility that an increase in intracellular Na\(^+\) concentration itself may be sufficient for the Rho response. To test this possibility, we incubated nystatin-permeabilized cells in various media in which Na\(^+\) concentration was increased at the expense of K\(^+\) concentration, thereby keeping the overall ionic strength at a constant (isosmotic) level. Elevating Na\(^+\) concentration from 10 to 140 mM did not activate Rho (Fig. 1D). In fact, at the higher Na\(^+\) concentrations (and at lower intracellular K\(^+\) concentration), a tendency toward lower Rho activity was observed, but it did not reach statistical significance. This latter finding is consistent with a recent report (39) showing that K\(^+\) depletion may inhibit Rho. Collectively, these data show that the potential triggering factor in Rho activation is elevated ionic strength and not a rise in intracellular Na\(^+\), K\(^+\), or Cl\(^-\) concentration.

In the following experiments, we examined whether a decrease in cell volume without an increase in intracellular ionic strength is also sufficient to stimulate Rho. Two strategies were used to assess this possibility. First, we incubated the cells in Iso-NMDG, a medium composed of large and impermeable ions. Under these conditions, the cell volume is expected to decrease because of the efflux of monovalent ions down their chemical gradient, followed by osmotically obliged water. Cells bathed in this solution exhibited a rapid, robust drop in their volume (Fig. 2, column 7), confirming that they indeed underwent strong isosmotic shrinkage. Concomitant with this volume reduc-
tion, a marked Rho activation occurred (2.82 ± 0.37-fold, n = 3: Fig. 4A). As an alternative approach, LLC-PK1 cells were permeabilized to monovalent ions with the use of nystatin and subsequently treated with 300 mM sucrose. This treatment induces a substantial decrease in cell volume (Fig. 2, column 8) but allows the free exit of monovalent ions, thereby ensuring the constancy of the intracellular ionic strength. This maneuver also resulted in increased Rho activation (2.40 ± 0.40-fold, n = 8; Fig. 4B). Taken together, these results show that cell shrinkage per se is also capable of inducing Rho activation, without increased salinity, and irrespective of whether it was induced under isosmotic (NMDG) or hyperosmotic (nystatin + sucrose) conditions. Moreover, increased ionic strength or cell shrinkage, produced by the same technique (nystatin permeabilization), each activated Rho. The isolated volume effect, however, appears to be somewhat less than the salt-induced response.

Role of the Rho-Rho Kinase Pathway in Hyperosmotic Stress-Induced Phosphorylation of MLC

In subsequent studies, we tested the effect of hyperosmotic exposure on MLC phosphorylation in intact LLC-PK1 cells. The differentially phosphorylated
forms of MLC were separated from lysates obtained from iso- or hypertonically treated cells with the use of urea-glycerol PAGE, and the abundance of the non-, mono- and diphosphorylated forms (as the percentage of total myosin in the same sample) was determined (Fig. 5, A and D). In agreement with our previous findings (31), LLC-PK1 cells expressed two isoforms of MLC, both of which can be differentially phosphorylated. This conclusion is supported by the finding that when whole cell lysates were analyzed using SDS-PAGE (which does not separate differentially phosphorylated versions), MLC was visualized as a doublet (Fig. 5B). The existence of multiple MLC isoforms has been reported in other cell types as well (16, 40).

Accordingly, on urea-glycerol PAGE, MLC obtained from LLC-PK1 cells could be separated into three doublets (6 bands): the two uppermost bands corresponded to the nonphosphorylated forms, the middle bands to the monophosphorylated forms (pMLC), and the lowermost bands to the diphosphorylated forms (ppMLC). The identity of these bands was verified by controls: the middle doublet was selectively visualized with an anti-phospho-Ser19-specific MLC antibody (Fig. 5A, left), whereas induction of maximal phosphorylation using the phosphatase inhibitor calyculin-A resulted in the exclusive labeling of the lowermost doublet (Fig. 5C).

In isotonically treated cells, the majority of MLC (~70%) was found in the nonphosphorylated form, with ~20% as pMLC and ~10% as ppMLC. Hyperosmotic exposure induced a substantial increase in both the mono- and the diphosphorylated forms, provoking a 1.6- and 2.0-fold rise, respectively (Fig. 5D). Next, we tested whether ROK, a downstream effector of Rho and a major regulator of MLC phosphorylation, plays a role in the hyperosmotic MLC response. We quantified the effect by determining the changes in ppMLC, because this marker gave the most robust and consistent response when different batches of the anti-MLC antibody were used. Y-27632, a potent and selective ROK inhibitor, dramatically reduced hyperosmotic MLC phosphorylation (Fig. 6A). Under isotonic conditions, ROK inhibition suppressed MLC phosphorylation below the basal level. In hypertonically treated cells, Y-27632 prevented the rise in MLC phosphorylation beyond the levels observed in untreated, isotonic cells. Thus, in the presence of Y-27632, the net phosphorylation of MLC in hypertonic cells was not more than in untreated controls. Nevertheless, comparing the phosphorylation levels under iso- and hypertonic conditions showed that hypertonicity was still able to induce an increase in MLC phosphorylation in the presence of Y-27632. Although the possibility of an incomplete inhibitory effect cannot be fully excluded, this finding suggests that a component of the process is ROK independent.

Because ROK activation may occur through Rho-independent mechanisms as well (49), we wanted to substantiate that the Y-27632-sensitive MLC phosphorylation was caused by upstream Rho activation. Therefore, cells were pretreated with ToxB, a cell-impermeant enzyme that ADP-glucosylates and thereby inactivates members of the Rho family (17). ToxB prevented the hyperosmolarity-induced MLC phosphorylation (Fig. 6B).

ROK may directly phosphorylate MLC (48, 50), but it may also potentiate the effect of other MLC-phosphorylating enzymes, predominantly MLCK, by inhibiting MLCPase (21). Because MLCK has been implicated in osmotically provoked MLC phosphorylation (23, 34), we assessed its contribution using ML-7, a potent MLCK inhibitor. Interestingly, hypertonic exposure remained able to provoke a sizable rise in MLC phosphorylation over the basal level in LLC-PK1 cells, even in the presence of a high concentration (75 μM) of ML-7.

**Fig. 5.** Hyperosmolarity induces both mono- and diphosphorylation of myosin light chain (MLC) in LLC-PK1 cells. A: LLC-PK1 cells were treated iso- or hypertonically (+300 mM sucrose) for 15 min and lysed in an acetone-TCA extraction buffer. The precipitated proteins were then resolubilized and subjected to nondenaturing urea-glycerol PAGE, followed by Western blotting (WB) with anti-MLC antibody (MLC) or anti-phospho-Ser19-specific MLC antibody (pMLC) in parallel. B: whole cell lysate from untreated LLC-PK1 cells was subjected to 15% SDS-PAGE, followed by Western blotting with anti-MLC antibody. C: LLC-PK1 cells were treated in an isotonic solution in the absence (control) or presence of calyculin A (10 nM) for 10 min. Differentially phosphorylated forms of MLC were detected by urea-glycerol PAGE as in A. D: the percentages of nonphosphorylated (MLC), monophosphorylated (pMLC), and diphosphorylated (ppMLC) forms of MLC under isotonic and hypertonic conditions were determined by densitometric analysis (n = 8).
Fig. 6. Rho/Rho kinase (ROK) pathway plays a central role in hypertonic MLC phosphorylation. LLC-PK1 cells were preincubated for 20 min in the absence or presence of 20 μM Y-27632 (A, left) or 75 μM ML-7 (C, left) or for 3–4 h in the absence or presence of 4 pM Clostridium difficile toxin B (ToxB) (B). Subsequently, the cells were treated iso- or hypertonically (+300 mM sucrose) for 15 min under the same inhibitor conditions. MLC was then precipitated and separated by urea-glycerol PAGE and Western blotted with anti-MLC antibody. Quantitative analysis of the effect of Y-27632 (A, right) and ML-7 (C, right) on the level of ppMLC under isotonic and hypertonic conditions was determined by densitometry (n = 3–5).

Uncoupling Between the ROK-Dependent MLC Phosphorylation and the Osmotic NKCC Activation

Previous studies have suggested that the hyperosmotically provoked MLC phosphorylation might be an upstream requirement for the shrinkage-induced NKCC activation (23, 34). Defining a central pathway for osmotic MLC phosphorylation provided us with a new opportunity to address two important questions: Is ROK involved in the osmotic activation of NKCC? Is an overall increase in MLC phosphorylation a prerequisite for the hypertonic NKCC activation?

To address these issues, we determined NKCC activity under iso- and hypertonic conditions by measuring the ouabain-insensitive, bumetanide-sensitive 86Rb uptake (3). In control cells, hyperosmolarity induced strong (~4-fold) activation of NKCC. Pretreatment of the cells with Y-27632 had no effect on the isotonic NKCC activity and only marginally reduced (<10%) the hyperosmotic NKCC stimulation (Fig. 7A). In sharp contrast, ML-7 reduced the isotonic NKCC activity and almost completely prevented the hyperosmotic activation of the cotransporter (Fig. 7B). It is noteworthy that, under identical experimental conditions, Y-27632 had a strong inhibitory effect on MLC phosphorylation, whereas ML-7 had no effect on the basal phosphorylation and exerted only a partial mitigating effect under hypertonic conditions (Fig. 6, A and C). Thus the order of potency of the two inhibitors was diametrically opposed for the MLC response and the NKCC activation. Similar inhibitory patterns were observed in porcine aortic endothelial cells as well (data not shown). These results unambiguously show that ROK does not play an important role in the hypertonic NKCC stimulation. Moreover, a substantial part of the osmotic MLC phosphorylation and NKCC activation can be pharmacologically uncoupled, raising the possibility that either the coupling between these processes is not obligate or only a separate ROK-independent MLC pool regulates NKCC activity (see DISCUSSION).

DISCUSSION

Hyperosmolarity-Induced Activation of Rho

Accumulating evidence suggests that members of the Rho family of small GTPases may be involved in osmotic signaling, but little is known about their roles in volume sensing and effector responses. The two central findings of the present study are that Rho is activated by hyperosmotic stress and that this process plays a central role in the osmotically induced phos-
the presence of 1.5 M/H11001 100 mM sucrose) in cells were then treated iso- or hypertonically (or hypertonic treatment, 1 mM ouabain was added to the cells. The Y-27632 (M ML-7 (B) or 75 μM ML-7 (B) for 20 min. Two minutes before iso-

The mechanisms whereby hyperosmotic challenge activates Rho remain to be clarified. We have identified two potent triggering factors: an increase in the total ionic force and a decrease in cell volume. Each of these parameters is able to separately turn on Rho signaling. It remains to be determined whether similar or distinct upstream pathways mediate the effect of these inputs. When induced by salt, Rho activation correlates with the overall ionic strength, whereas changes in the individual ion concentrations do not seem to be critical. The salt sensitivity appears to be a general feature in the regulation of various members of the Rho family, because our previous studies (29) showed that Rac was also activated by high ionic strength. Conceivably, high salinity may act on upstream regulators common to various members of the Rho family.

Conceptually, increased Rho activity may result from activation of guanine nucleotide exchange factors (GEFs), the inhibition of GTPase activating proteins (GAPs), and the inactivation of GDP dissociation inhibitors (GDI) (12). Given the abundant numbers of proteins in each category, the identification of the responsible regulators is a challenging task. Nevertheless, one of the candidate upstream signals for the volume-dependent Rho response could be the activation of certain Src family kinases. Our previous studies (18, 19) have shown that Fyn is stimulated by hyperosmotic stress and mediates downstream cytoskeletal and genetic effects. Specifically, Fyn participates in the shrinkage-induced phosphorylation of the F-actin binding protein cortactin (19) and in the induction of the osmotic response element (24). Intriguingly, Fyn is known to phosphorylate Vav (15), an important GEF for Rho, and the existence of a positive feedback loop between Fyn and Rho has recently been suggested (6).

Potentially, osmosenitive Rho GEF-activating mechanisms include changes in inositol phosphates (9) and the rearrangement of the microtubule apparatus (5). Microtubule disassembly was shown to increase Rho activity by liberating GEF-H1 (27). However, we consider this mechanism less likely, because the microtubule-stabilizing drug taxol failed to prevent the hypertonic activation of Rho in our experiments (not shown). Nevertheless, further studies are warranted to investigate these possibilities.

Hyperosmotic Phosphorylation of MLC

Previous studies have implicated MLCK (23) and PKC (46) in osmotic MLC phosphorylation. We propose that the Rho/ROK pathway also participates in this process. This view is supported by our findings that 1) Rho, a direct activator of ROK, is stimulated by hypertonicity; 2) the Rho family inhibitor ToxB eliminates the basal and prevents the osmotic MLC phosphorylation; and 3) the ROK inhibitor Y-27632 reduces MLC phosphorylation in iso- and hypertonically treated

![Fig. 7. Differential effect of Y-27632 and ML-7 on the hyperosmotic activation of the Na+–K+–Cl– cotransporter (NKCC). LLC-PK1 cells were pretreated in Iso-NaCl in the absence or presence of 20 μM Y-27632 (A) or 75 μM ML-7 (B) for 20 min. Two minutes before iso-

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Phosphorylation of MLC via the Rho/ROK pathway. Interestingly, previous studies have implicated Rho in cellular responses to hypotonicity or the concomitant cell swelling. Inhibition of the Rho/ROK pathway by C3 transferase or Y-27632 have been reported to decrease the hypotonic stress-induced activation of volume-regulated anion channels (VRAC) in epithelial (47), vascular endothelial (33), and neuroblastoma cells (11) and to prevent the hypotonicity-provoked ATP release from aortic endothelium (25). Furthermore, overexpression of a constitutive active Rho mutant (RhoAV14) has been found to potentiate the rate of regulatory volume decrease in swollen NIH/3T3 fibroblasts (36). On the other hand, constitutively active versions of Rho or ROK did not activate VRAC under isonic conditions, and they did not facilitate the swelling-induced activation of the channel. Most importantly, hypotonicity failed to stimulate Rho (7). Collectively, these findings suggest that Rho plays a permissive rather than an active role in the mediation of swelling-induced responses (7). This essential, albeit indirect role is likely related to the fact that intact Rho signaling is necessary for maintaining cytoskeletal integrity, which in turn has a major impact on the orchestration and modulation of volume-dependent responses (38). In contrast to this housekeeping function, our studies assign an active and direct role for Rho in hyperosmotic signaling. Rho activity accurately mir-
cells. The contribution of the Rho/ROK pathway is consistent with the involvement of MLCK, because ROK, via the inhibition of MLCPase, can unmask a constitutive MLCK activity, which manifests in a net increase in MLC phosphorylation; that is, hypotonicity may lead to MLCK-dependent MLC phosphorylation without activating MLCK. Thus Rho/ROK-dependent inhibition of MLCPase may be a significant mechanism facilitating osmotic MLC phosphorylation. Consistent with this notion, we found that pharmacological phosphatase inhibition by calyculin A led to enhanced MLC phosphorylation, indicating constitutive MLCPase activity. In addition, our results suggest that ROK directly participates in osmotic MLC phosphorylation because 1) inhibition of MLCK by ML-7 does not abolish the hyperosmotic phosphorylation of MLC, and 2) Y-27632 has a stronger suppressive effect than ML-7. Y-27632 dramatically reduces basal MLC phosphorylation and prevents its increase above the isotonic level in hypertonically stimulated cells. Nevertheless, hyperosmotic stress remains able to increase MLC phosphorylation in Y-27632-treated cells, implying that the ROK-dependent mechanisms (phosphatase inhibition and direct phosphorylation) cannot be fully responsible for the phenomenon and that osmotic activation of other MLC-phosphorylating enzymes contributes to the effect.

Relationship Between MLC Phosphorylation and Osmotic Regulation of NKCC

The notion that cell contractility, presumably through the phosphorylation state of MLC, regulates NKCC was based on findings that pharmacological agents interfering with MLCK (ML-7, ML-9) or myosin ATPase (2,3-butanedione-2-monoxime, BDM) inhibited the agonist-induced (phenylephrine, forskolin) activation of the cotransporter. Because ML-7 did not prevent the hyperosmotically induced phosphorylation of NKCC per se (23), the involvement of other targets, predominantly MLC, appeared an attractive mechanism. Intriguingly, our studies show that a strong suppression of the osmotically induced MLC phosphorylation leaves NKCC activation intact, suggesting that the two phenomena may not be tightly coupled. We would like to consider two mutually nonexclusive possibilities when interpreting these results. First, there is ambiguity in the literature as to whether the applied inhibitors exert their effect on NKCC necessarily or exclusively by interfering with MLC phosphorylation (20). Whereas in a few cell types low concentrations of ML-7 are inhibitory (26, 34), in other cells the concentration required to inhibit NKCC (or the Na+/H+ exchanger) is at least two orders of magnitude higher than necessary for in vitro inhibition of MLCK (14, 23, 42). Furthermore, ML-7 has been shown to stimulate the K+/Cl− cotransporter, a hypotonically-induced ion carrier, independently of MLCK inhibition (20). Moreover, MLCK has been reported to alter ion transporters independently of MLC phosphorylation (41). It is thus conceivable that the reduction in NKCC activity and MLC phosphorylation are parallel but not causally linked processes. Similar arguments hold for BDM, which is a well-known “chemical phosphatase” (43) and may induce the dephosphorylation of volume-regulatory proteins involved in NKCC activation. In agreement with this possibility, Akar et al. (2) observed that inhibition of contraction did not correlate precisely with NKCC inhibition, suggesting that the two processes are not tightly coupled.

An alternate explanation for our findings is the existence of spatially distinct and differentially regulated MLC pools, which may serve separate functional roles. This possibility is very attractive in light of recent observations that central and peripheral stress fibers appear to be regulated by different MLC-phosphorylating enzymes. In 3T3 fibroblasts, ROK was found to be involved in MLC phosphorylation predominantly in the center of the cell, whereas MLCK acted mostly at the periphery (48). Interestingly, in smooth muscle cells the opposite organization was reported (32). Irrespective of cell type-specific differences, the presence of distinct phospho-MLC pools may account for apparent discrepancies between the total MLC response and the MLC-dependent NKCC regulation. Thus, whereas a substantial portion of MLC phosphorylation is ROK dependent, the ROK-sensitive pool may not be involved in NKCC regulation. Interestingly, a similar pattern is seen in the case of hypertonic activation of the Na+/H+ exchanger: whereas high doses of ML-7 inhibited this response in certain cells (42), Y-27632 had no effect on the process (37, 42).

Hyperosmolarity may alter the spatial distribution of Rho, ROK, MLCK, or myosin. Indeed, Y-27632-sensitive peripheral translocation of myosin was recently reported in Ehrlich ascites cells (37). Moreover, we found that hypertonicity reduced F-actin in central stress fibers but induced the accumulation of actin and cortactin at the periphery, presumably in a Rac-and Cdc42-dependent manner (8). Interestingly, cortactin was recently described to bind MLCK (10), a finding that offers an attractive potential mechanism for MLCK recruitment near the membrane. This scenario may explain why ToxB, which inhibits all members of the Rho family, exerted an even stronger inhibition on MLC phosphorylation than Y-27632. Regrettably, with the available antibodies we could not unambiguously localize phospho-MLC accumulation or verify potential changes in ROK distribution in osmotically shrunken cells. Temporal and spatial resolution of these processes would further our understanding about the volume-dependent regulation of the cytoskeleton.

Finally, it is worth noting that although MLC phosphorylation may be required for osmotic NKCC activation, it may not be sufficient. The involvement of other kinases, some of which may directly phosphorylate the transporter, is indicated by the findings that inhibition of PKC or ERK reduced the osmotic activation of NKCC (30). Moreover, c-Jun NH2-terminal kinase, an osmosensitive enzyme and downstream target of Rac
and Cdc42, has been reported to phosphorylate NKCC in vitro (22).

In summary, we have shown that Rho is activated by hyposmotic shock, and this reaction seems to be one of the mechanisms underlying the hypertonicity-induced MLC phosphorylation. The ROK-dependent MLC phosphorylation may be a key step in the reinforcement of the cytoskeleton in the challenged cell, but it does not participate in the ensuing activation of NKCC.

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DISCLOSURES

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