The Basolateral NHE1 Na\(^+\)/H\(^+\) Exchanger Regulates Transepithelial HCO\(_3^-\) Absorption through Actin Cytoskeleton Remodeling in Renal Thick Ascending Limb*

Received for publication, September 17, 2004, and in revised form, December 20, 2004 Published, JBC Papers in Press, January 11, 2005, DOI 10.1074/jbc.M410719200

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In the renal medullary thick ascending limb (MTAL), inhibiting the basolateral NHE1 Na\(^+\)/H\(^+\) exchanger with amiloride or nerve growth factor (NGF) results secondarily in inhibition of the apical NHE3 Na\(^+\)/HCO\(_3^-\) exchanger, thereby decreasing transepithelial HCO\(_3^-\) absorption. MTALs from rats were studied by in vitro microperfusion to identify the mechanism underlying cross-talk between the two exchangers. The basolateral addition of 10 μM amiloride or 0.7 mM NGF decreased HCO\(_3^-\) absorption by 27–32%. Jasplakinolide, which stabilizes F-actin, or latrunculin B, which disrupts F-actin, decreased basal HCO\(_3^-\) absorption by 30% and prevented the inhibition by amiloride or NGF. Jasplakinolide had no effect on HCO\(_3^-\) absorption in tubules bathed with amiloride or a Na\(^+\)-free bath to inhibit NHE1. Jasplakinolide and latrunculin B did not prevent inhibition of HCO\(_3^-\) absorption by vasopressin or stimulation by hyposmolality, factors that regulate HCO\(_3^-\) absorption through primary effects on apical Na\(^+\)/H\(^+\) exchange. Treatment of MTALs with amiloride or NGF for 15 min decreased polymerized actin with no change in total cell actin, as assessed both by fluorescence microscopy and by actin Triton X-100 solubility. Jasplakinolide prevented amiloride-induced actin remodeling. Vasopressin, which inhibits HCO\(_3^-\) absorption by an amount similar to that observed with amiloride and NGF but does not act via NHE1, did not affect cellular F-actin content. These results indicate that basolateral NHE1 regulates apical NHE3 and HCO\(_3^-\) absorption in the MTAL by controlling the organization of the actin cytoskeleton.

* This work was supported by National Institutes of Health Grant DK-38217 and by a grant from the John Sealy Memorial Endowment Fund for Biomedical Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: NHE, Na\(^+\)/H\(^+\) exchanger (the number following “NHE” refers to the specific isoform); MTAL, medullary thick ascending limb; NGF, nerve growth factor; AVP, arginine vasopressin; PBS, phosphate-buffered saline.

Na\(^+\)/H\(^+\) exchangers (NHEs)

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ezrin (7, 25, 26), and cytoskeletal interactions play a role in the regulation of NHE3 by factors such as cAMP and endothelin (26–29). In an antiporter-deficient fibroblast cell line transfected with NHE3, either stabilizing or depolymerizing actin filaments markedly inhibited NHE3 by decreasing its intrinsic activity (25). When considered in the context of epithelial cells such as the MTAL that express NHE1 in the basolateral membrane and NHE3 in the apical membrane, the above findings raise the possibility that the cytoskeleton could mediate cross-talk between the two exchangers, whereby NHE1 could induce changes in the actin network that in turn modulate NHE3.

The purpose of the present experiments was to investigate the role of the actin cytoskeleton in transepithelial HCO₃⁻ absorption in the MTAL and to determine whether the cytoskeleton mediates the regulatory interaction between the basolateral NHE1 and apical NHE3 Na⁺/H⁺ exchangers. The results demonstrate that NHE1 regulates the organization of the actin cytoskeleton in the MTAL and that actin remodeling is involved in mediating NHE1-induced regulation of apical NHE3 and HCO₃⁻ absorption.

**EXPERIMENTAL PROCEDURES**

**Tubule Perfusion and Measurement of Net HCO₃⁻ Absorption—**

MTALs from male Sprague-Dawley rats (50–100-g body weight; Tac- onic, Germantown, NY) were isolated and perfused in vitro as described (19, 30). Tubules were dissected from the inner stripe of the outer medulla at 10 °C in control bath solution (see below), transferred to a bath chamber on the stage of an inverted microscope, and mounted on concentric glass pipettes for perfusion at 37 °C. In most experiments, the tubules were perfused and bathed in control solution that contained 146 mM Na⁺, 4 mM K⁺, 122 mM Cl⁻, 25 mM HCO₃⁻, 2 mM Ca²⁺, 1.5 mM Mg²⁺, 2.0 mM phosphate, 1.2 mM SO₄²⁻, 1.0 mM citrate, 2.0 mM lactate, and the osmolality was 295 mosmol/kg H₂O. In one series of experiments (Fig. 4B), Na⁺ in the bath solution was replaced completely with N-methyl-d-glucamine (19, 20). Hyposmotic solutions (245 mosmol/kg H₂O) (Figs. 3B and 6B) were produced by removing 25 mM NaCl from the control solution or by removing 50 mM mannitol from control solution in which 50 mM mannitol replaced 25 mM NaCl (15). Bath solutions contained 0.2 g/100 ml fatty acid-free bovine albumin. All solutions were equilibrated with 95% O₂, 5% CO₂ and were pH 7.45 at 37 °C. Experimental agents were added to the bath solutions as described under “Results.” Jaspilkinolide and Itrunuculin B were prepared as stock solutions in dimethyl sulfoxide and diluted into bath solutions as needed. The results are expressed as a percentage of the control value measured in the same experiment. Mean values for repeat experiments were used for statistical analysis.

**Actin Detergent Solubility—**

The relative amounts of actin in Triton X-100-soluble and -insoluble fractions were determined using established protocols (25, 31, 32) and a previously described inner stripe tissue preparation (33, 34). In brief, thin strips of tissue were dissected at 4 °C from the inner stripe of the outer medulla, the region of the kidney highly enriched in MTALs. The strips were divided into 2–4 samples of equal amount and then incubated in vitro at 37 °C in the same solutions used for HCO₃⁻ transport experiments (33, 34). The specific protocols for incubation are given under “Results.” After incubation, the tissue samples were homogenized in ice-cold buffer (150 mM NaCl, 30 mM Hepes, pH 7.4) and then incubated for 3 h at 4 °C after the addition of an equal volume of Triton X-100 extraction buffer (final composition 5 mM imidazole, 37.5 mM KCl, 2.5 mM MgCl₂, 0.5 mM EDTA, 1% Triton X-100, and protease inhibitor mixture (Sigma), titrated to pH 7.2) (32). The lysates were centrifuged at 100,000 x g at 4 °C for 45 min. The supernatants were then collected, and the pellets were resuspended in a volume equivalent to that of the supernatant. Proteins were treated with Laemmli sample buffer, separated by SDS-PAGE on 12% gels, and transferred to polyvinylidene difluoride membranes (34). The membranes were blocked with TBS, 0.1% Tween 20, and 1% bovine serum albumin and exposed to monoclonal anti-actin antibody (AC-40; 1:1000; Sigma) for 2 h at room temperature. Immunoreactive bands were detected by ECL (Amersham Biosciences) after application of horseradish peroxidase-conjugated goat anti-mouse secondary antibody. Relative band intensities were quantified by densitometry (34), and the results are expressed as the percentage of total cell actin present in the detergent-soluble and -insoluble fractions. Equal amounts of total cellular actin in different experimental conditions were verified by immunoblotting of HCO₃⁻ absorbing tissue lysates. Peroxidase-labeled NGF decreased HCO₃⁻ absorption by 32%, from 10.4 ± 0.8 pmol/min/mm (p < 0.001) (Fig. 1A). The bath addition of 0.7 nM NGF decreased HCO₃⁻ absorption by 32%, from 14.4 ± 0.6 to 9.8 ± 0.4 pmol/min/mm (p < 0.025) (Fig. 1B). Both effects were complete within 15 min and were reversible. These data confirm previous results demonstrating that bath amiloride or NGF inhibits HCO₃⁻ absorption in the MTAL through inhibition of the basolateral NHE1 Na⁺/H⁺ exchanger (18–20).

**RESULTS**

**Bath Amiloride and NGF Inhibit HCO₃⁻ Absorption—**

Adding 10 μM amiloride to the bath decreased HCO₃⁻ absorption in isolated rat MTALs by 27%, from 14.2 ± 0.7 to 10.4 ± 0.8 pmol/min/mm (p < 0.001) (Fig. 1A). The bath addition of 0.7 nM NGF decreased HCO₃⁻ absorption by 32%, from 14.4 ± 0.6 to 9.8 ± 0.4 pmol/min/mm (p < 0.025) (Fig. 1B). Both effects were complete within 15 min and were reversible. These data confirm previous results demonstrating that bath amiloride or NGF inhibits HCO₃⁻ absorption in the MTAL through inhibition of the basolateral NHE1 Na⁺/H⁺ exchanger (18–20).

**Effects of Jaspilkinolide on HCO₃⁻ Absorption—**

To investigate the role of the cytoskeleton in HCO₃⁻ absorption, we examined the effects of jaspilkinolide, a membrane-permeant cyclic peptide that binds and stabilizes actin filaments and promotes actin polymerization (35). Adding 0.05 μM jaspilkinolide to the bath decreased HCO₃⁻ absorption by 32%, from 14.8 ± 0.3 to 10.0 ± 0.4 pmol/min/mm (p < 0.001) (Fig. 2A). This inhibition was observed within 15–20 min after the addi-
tion of jasplakinolide and was reversible. In MTAL bathed with 0.05 μM jasplakinolide for 25–30 min, the addition of either 10 μM amiloride or 0.7 nM NGF to the bath had no effect on HCO₃⁻ absorption (Fig. 2, A and B). These results suggest that the basal rate of HCO₃⁻ absorption and the inhibition of HCO₃⁻ absorption by bath amiloride and NGF depend on the state of the actin cytoskeleton.

To assess the specificity of jasplakinolide’s actions on the regulation of HCO₃⁻ absorption, we examined the effects of vasopressin (AVP) and hyposmolality. These factors were studied because AVP inhibits and hyposmolality stimulates HCO₃⁻ absorption in the MTAL through primary effects on the apical Na⁺/H⁺ exchanger (13, 15, 30, 36), contrary to bath amiloride and NGF, which act primarily on the basolateral Na⁺/H⁺ exchanger. In MTAL bathed with 0.05 μM jasplakinolide for 25–50 min, adding 10⁻¹⁰ M AVP to the bath decreased HCO₃⁻ absorption by 43%, from 11.4 ± 0.5 to 6.5 ± 0.9 pmol/min/mm (p < 0.001; Fig. 3A), and hyposmolality increased HCO₃⁻ absorption from 10.5 ± 0.7 to 16.0 ± 1.4 pmol/min/mm (p < 0.025; Fig. 3B). These effects are similar to those observed in the absence of jasplakinolide (15, 30). Thus, the regulation of HCO₃⁻ absorption by AVP and hyposmolality is preserved in the presence of the F-actin modifier. These results demonstrate that the effect of jasplakinolide to block HCO₃⁻ transport regulation is specific for factors (bath amiloride and NGF) that act via the basolateral NHE1 Na⁺/H⁺ exchanger.

The preceding results suggest that jasplakinolide and factors that inhibit NHE1 may decrease HCO₃⁻ absorption through a common mechanism, namely modification of the actin cytoskeleton. If this hypothesis is correct, then the effect of jasplakinolide to inhibit HCO₃⁻ absorption should be diminished under conditions in which basolateral Na⁺/H⁺ exchange is inhibited. To test this, we examined the effect of jasplakinolide in the presence of bath amiloride or in the absence of bath Na⁺, two conditions that inhibit basolateral Na⁺/H⁺ exchange (19, 20).

In MTAL bathed with 10 μM amiloride, adding 0.05 μM jasplakinolide to the bath decreased HCO₃⁻ absorption only by 11%, from 11.0 ± 0.6 to 9.7 ± 0.8 pmol/min/mm (p < 0.005) (Fig. 4A). When compared with the effect of jasplakinolide in control experiments (Fig. 2A), 10 μM bath amiloride reduced the inhibition by jasplakinolide by 70% (p < 0.05). In tubules studied in a Na⁺-free bath, adding 0.05 μM jasplakinolide to the bath had no effect on HCO₃⁻ absorption (14.4 ± 0.8 pmol/min/mm, 0 Na⁺ bath versus 14.3 ± 1.0 pmol/min/mm, 0 Na⁺ bath + jasplakinolide; p = not significant) (Fig. 4B). Thus, the effect of jasplakinolide to inhibit HCO₃⁻ absorption was virtually eliminated under two different conditions in which basolateral Na⁺/H⁺ exchange was inhibited. These results support the view that jasplakinolide and NHE1 regulate HCO₃⁻ absorption through a common mechanism involving modification of the actin cytoskeleton.

Effects of Latrunculin B on HCO₃⁻ Absorption—To investigate further the role of the actin cytoskeleton in HCO₃⁻ absorption, we examined the effects of latrunculin B. This compound sequesters monomeric actin and induces F-actin depolymerization (37), actions opposite to the F-actin-stabilizing effects of jasplakinolide. Adding 1 μM latrunculin B to the bath decreased HCO₃⁻ absorption by 31%, from 14.9 ± 0.8 to 10.3 ± 0.9 pmol/min/mm (p < 0.005) (Fig. 5A). The inhibition by latrunculin B occurred after a time delay of 70–80 min. This time course parallels the effect of latrunculin B on the actin cytoskeleton (see below). The HCO₃⁻ absorption rate returned to its initial control value within 30–40 min after the removal of latrunculin B from the bath solution. In MTALs bathed with 1 μM latrunculin B for 80–100 min, adding 10 μM amiloride or 0.7 nM NGF to the bath had no effect on HCO₃⁻ absorption (Fig. 5, B and C). Thus, either disrupting F-actin with latrunculin B or stabilizing F-actin with jasplakinolide prevents inhibition of HCO₃⁻ absorption by bath amiloride and NGF (see “Discussion”). These results support further the hypothesis that regulation of HCO₃⁻ absorption by bath amiloride and NGF involves the actin cytoskeleton.

As with jasplakinolide, the specificity of latrunculin B action was tested using AVP and hyposmolality. In MTAL bathed with 1 μM latrunculin B for 100 min, AVP decreased HCO₃⁻ absorption from 9.3 ± 0.7 to 4.2 ± 0.3 pmol/min/mm (p < 0.001) (Fig. 6A), and hyposmolality increased HCO₃⁻ absorption from 9.9 ± 1.0 to 13.0 ± 1.1 pmol/min/mm (p < 0.001) (Fig. 6B). Both effects were reversible. These results demonstrate further that altering the actin cytoskeleton blocks selectively the regulation of HCO₃⁻ absorption by factors that act via basolateral NHE1.

Effects of Colchicine on HCO₃⁻ Absorption—To determine whether the regulation of HCO₃⁻ absorption via basolateral Na⁺/H⁺ exchange involves cytoskeletal structures in addition to F-actin, we examined the effects of colchicine, an inhibitor of microtubule assembly. In MTAL bathed with 50 μM colchicine for 70–80 min, the addition of 10 μM amiloride to the bath decreased HCO₃⁻ absorption by 38%, from 12.4 ± 1.0 to 7.7 ± 0.6 pmol/min/mm (p < 0.005) (Fig. 7). A similar or less severe colchicine treatment has been shown to block other microtubule-dependent processes in renal cells (38–40). Thus, these results suggest that the effect of bath amiloride to inhibit HCO₃⁻ absorption via basolateral Na⁺/H⁺ exchange is not dependent on microtubules.

Effects of Inhibitors of NHE1 on the Actin Cytoskeleton—To examine more directly possible interactions between NHE1 and the cytoskeleton, F-actin was studied in microdissected MTALs by Alexa 488-phalloidin staining and confocal fluorescence microscopy (Fig. 8). Tubules were optically sectioned, three-dimensional images were constructed, and the fluorescence intensity of phalloidin staining normalized for tubule volume was quantified. MTALs exhibit F-actin staining along the inner surface of the plasma membranes (cortical actin), a diffuse F-actin network throughout the cytoplasm, and a dense annular bundle of actin filaments surrounding the apical cell pole (actin belt of the zonula adherens) (Fig. 8, A and C). Treatment with either 10 μM amiloride or 0.7 nM NGF for 15 min decreased the intensity of fluorescence staining by 30%
This decrease is attributable to a decrease in cellular F-actin content rather than to a change in cell volume based on the following observations: 1) treatment with amiloride or NGF has no effect on cell volume (Metamorph analysis) (19, 20); 2) amiloride and NGF cause a similar decrease in fluorescence intensity when normalized per mm of tubule length (indicating decreased F-actin per cell); and 3) decreased cellular F-actin is confirmed independently by measurement of actin detergent solubility (see below). At the cellular level, amiloride or NGF treatment decreased F-actin lining the basal cell surface, reduced cortical actin underlying the cell membranes, and decreased the cytoplasmic F-actin network (Fig. 8C). In contrast, treatment for 15 min with 10 μM AVP, which inhibits HCO₃⁻ absorption via a primary effect on the apical Na⁺/H⁺ exchanger, had no effect on F-actin fluorescence intensity (Fig. 8A and B). Thus, cytoskeletal remodeling was induced selectively by factors (amiloride and NGF) that inhibit HCO₃⁻ absorption through primary inhibition of basolateral NHE1. These results demonstrate that inhibitors of NHE1 induce reorganization of the cytoskeleton that involves a decrease in cellular F-actin.

Treatment with 1 μM latrunculin B for 60 min decreased fluorescence intensity (Fig. 8A and B), consistent with its action to depolymerize F-actin. Latrunculin B disrupted the cortical and cytoplasmic F-actin networks, with relative preservation of the annular F-actin bundle (see below). At the cellular level, amiloride or NGF treatment decreased F-actin lining the basal cell surface, reduced cortical actin underlying the cell membranes, and decreased the cytoplasmic F-actin network (Fig. 8C). In contrast, treatment for 15 min with 10⁻¹⁰ M AVP, which inhibits HCO₃⁻ absorption via a primary effect on the apical Na⁺/H⁺ exchanger, had no effect on F-actin fluorescence intensity (Fig. 8A and B). Thus, cytoskeletal remodeling was induced selectively by factors (amiloride and NGF) that inhibit HCO₃⁻ absorption through primary inhibition of basolateral NHE1. These results demonstrate that inhibitors of NHE1 induce reorganization of the cytoskeleton that involves a decrease in cellular F-actin.

Effects of Inhibitors of NHE1 on Actin Solubility—To examine further the interaction of NHE1 with the cytoskeleton, the relative amounts of actin in Triton X-100-soluble and -insoluble fractions were determined. Because actin filaments are resistant to mild detergent extraction, quantification of the fraction of Triton X-100-insoluble actin provides a measure of cellular F-actin content and the extent of actin polymerization (25, 31, 32). Inner stripe tissue was incubated in vitro using the same solutions and treatments as in HCO₃⁻ transport and fluorescence.
In the kidney, our experiments (0.05 M jasplakinolide for 15 min) showed that NHE1 induces reorganization of the cytoskeleton. Our data support a model (Fig. 11) in which inhibition of basolateral NHE1 induces a decrease in polymerized actin that inhibits the apical absorption of Na+/H+ exchangers. The results show that NHE1 regulates NHE3 and HCO₃⁻ absorption by controlling the organization of the actin cytoskeleton. Our data support a model (Fig. 11) in which inhibition of basolateral NHE1 induces a decrease in polymerized actin that inhibits the apical absorption of Na+/H+ exchangers.

The conclusion that NHE1 regulates NHE3 via the cytoskeleton is supported by both the effects of basolateral amiloride to induce F-actin remodeling and to inhibit HCO₃⁻ absorption.

**DISCUSSION**

Previously, we identified an important role for the basolateral NHE1 Na⁺/H⁺ exchanger in regulation of transepithelial HCO₃⁻ absorption in the MTAL. This involves a novel and paradoxical mechanism whereby inhibition of basolateral NHE1 results secondarily in the inhibition of apical NHE3, thereby decreasing Na⁺ absorption (18–20). These results provided the first evidence of a regulatory role for NHE1 in transepithelial transport in the kidney. In the present study, we examined the mechanism of cross-talk between the two Na⁺/H⁺ exchangers. The results show that NHE1 regulates NHE3 and HCO₃⁻ absorption by controlling the organization of the actin cytoskeleton. Our data support a model (Fig. 11) in which inhibition of basolateral NHE1 induces a decrease in polymerized actin that inhibits the apical NHE3 Na⁺/H⁺ exchanger.

**FIG. 5.** Latrunculin B (Lat B) blocks inhibition by bath amiloride and NGF. A, MTALs were studied in control solution, and then 1 μM latrunculin B was added to and removed from the bath solution. B and C, tubules were bathed with 1 μM latrunculin B for 80–100 min, and then 10 μM amiloride or 0.7 nM NGF was added to the bath solution. JHCO₃⁻, data points, lines, and p values are as in Fig. 1. NS, not significant. Mean values are given under "Results."

**FIG. 6.** Latrunculin B does not prevent regulation by AVP and hyposmolality. MTALs were studied in control solution with 1 μM latrunculin B in the bath for 100 min, and then 10⁻¹⁰ M AVP was added to the bath (A), or hyposmolality was produced in lumen and bath by removal of 50 mM mannitol (filled circles) or 25 mM NaCl (open circles) (B) (15). JHCO₃⁻, data points, lines, and p values are as in Fig. 1. Mean values are given under "Results."

Inner stripe tissue was incubated in control solution in the absence and presence of 0.05 M jasplakinolide for 15 min. The tissue was then either maintained in these solutions or treated with 10 μM amiloride for an additional 15 min. Treatment with jasplakinolide alone did not alter actin detergent solubility. However, jasplakinolide blocked completely the effects of amiloride to increase the Triton X-100-soluble fraction and to decrease the Triton X-100-insoluble fraction of cellular actin (Fig. 10, A and B). Thus, in the MTAL, jasplakinolide blocks both the effects of basolateral amiloride to induce F-actin remodeling and to inhibit HCO₃⁻ absorption.

**FIG. 7.** Colchicine (Colch) does not prevent inhibition by bath amiloride. MTALs were studied in control solution, and then 50 μM colchicine was added to the bath solution for 70–80 min. Amiloride (10 μM) was then added to and removed from the bath in the presence of colchicine. JHCO₃⁻, data points, lines, and p value are as in Fig. 1. Mean values are given under "Results."

**Data analysis**

Jasplakinolide has been shown to increase the fraction of Triton X-100-insoluble (polymerized) actin in some systems (25, 31, 32). However, these studies used much higher jasplakinolide concentrations (1–10 μM) and longer exposure times (45–120 min) than those used in our experiments (0.05 μM for 15–30 min). We used a relatively low dose of jasplakinolide that is above the IC₅₀ for F-actin binding (35), induced a rapid, stable, and reversible inhibition of HCO₃⁻ absorption, and blocked amiloride-induced F-actin rearrangement. The stabilizing effect of jasplakinolide on F-actin in our experiments did not result in a measurable change in actin Triton X solubility.

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sistent with actin modifiers and NHE1 inhibitors acting via a common mechanism; 3) jasplakinolide has no effect on HCO₃⁻/H₁¹₀₀₂⁻ absorption under conditions in which NHE1 is inhibited; 4) the actin modifiers block selectively the inhibition of HCO₃⁻/H₁¹₀₀₂⁻ absorption by factors that act via NHE1 (amiloride and NGF) but have no effect on regulation by factors that act primarily on NHE3 (AVP and hyposmolality); 5) inhibitors of NHE1 induce cytoskeletal remodeling that involves a decrease in polymerized actin, as verified using two independent methods of measurement (fluorescence microscopy and detergent solubility); 6) similar cytoskeletal changes are induced by two different NHE1 inhibitors, which argues against amiloride and NGF having nonspecific cytoskeletal effects; 7) AVP, which inhibits HCO₃⁻ absorption by an amount similar to that observed with NGF and amiloride but does not act through NHE1, had no effect on cellular F-actin content; and 8) jasplakinolide prevents amiloride-induced transport inhibition by blocking cytoskeletal remodeling.

Taken together, these findings indicate that reorganization of the cytoskeleton is necessary for NHE1-induced regulation of NHE3 and HCO₃⁻/H₁¹₀₀₂⁻ absorption. It is noteworthy that basal HCO₃⁻/H₁¹₀₀₂⁻ absorption is decreased, and NHE1-induced regulation is prevented, by agents that either disrupt or stabilize actin filaments. This suggests that the NHE1-induced regulation of NHE3 depends on dynamic actin rearrangement and not on a static actin network. These findings are consistent with previous results in AP-1 cells demonstrating that either disruption of F-actin with latrunculin B or stabilization of F-actin with jasplakinolide inhibits NHE3 activity (25). Our results show that inhibiting NHE1 in the MTAL shifts the steady state for

**FIG. 8. Amiloride and NGF induce actin cytoskeleton reorganization in the MTAL.** A, microdissected MTALs were incubated in vitro in control solution (Cont), 10 μM amiloride (Amil), 0.7 nM NGF, or 10⁻¹⁰ M AVP for 15 min or with 1 μM latrunculin B (Lat B) for 60 min. Results for 60-min controls (not shown) did not differ from 15-min controls. Tubules were fixed, permeabilized, and stained with Alexa 488-phalloidin to visualize F-actin. Three-dimensional images were constructed from longitudinal optical sections <0.4-μm thick. The arrows point to the annular F-actin bundle, which is most clearly visible in three-dimensional images in amiloride, NGF, and latrunculin B conditions. Images are representative of at least four tubules in each group. B, total fluorescence intensity normalized for tubule volume is presented for all treatment groups as the percentage of control value measured in the same experiment (see “Experimental Procedures”). Data are means ± S.E. for 4–8 independent experiments in each group. *, p < 0.05 versus control (analysis of variance). C, confocal images of z axis sections (0.35–0.36 μm) taken at the basal surface of cells on the bottom of the tubule (upper panels) and through a plane at the center of the tubule that shows a cross-sectional view of cells in the lateral tubule walls (lower panels). Images show a top-down view of sections obtained at equal distance from the tubule bottom for each experimental condition. Tubules were incubated in control solution, 10 μM amiloride, or 0.7 nM NGF for 15 min. Treatment with amiloride or NGF decreased the intensity of F-actin lining the basal cell surface (upper panels), underlying the cell membranes (lower panels, arrowheads), and in the cytoplasm (lower panels, c). Images are representative of at least six tubules of each type. Scale bars, 10 μm.
actin assembly toward F-actin depolymerization, which results in inhibition of NHE3.

NHE1 was identified unambiguously as the basolateral exchanger that regulates apical NHE3 and HCO$_3$/$H_1$ absorption through studies of MTALs from wild-type and NHE1 knockout mice (18). Of significance, genetic ablation of NHE1 produces regulatory changes in HCO$_3$/$H_1$ absorption that are strikingly similar to those observed with cytoskeletal modifiers. Both cause a decrease in the basal rate of HCO$_3$/$H_1$ absorption and eliminate inhibition of HCO$_3$/$H_1$ absorption by basolateral amiloride or NGF but do not prevent inhibition by AVP or stimulation by hyposmolality (18) (Figs. 2–6). The fact that NHE1 knockout and actin-modifying agents have virtually identical effects on HCO$_3$/$H_1$ absorption in five independent conditions (basal, amiloride, NGF, AVP, and hyposmolality) provides further strong support for the conclusion that NHE1 and the cytoskeleton are components of a common regulatory mechanism.

Several lines of evidence indicate that the effects of jasplakinolide and latrunculin B on HCO$_3$/$H_1$ absorption are attributable to their effects on the actin cytoskeleton and not to nonspecific metabolic or cytotoxic effects. First, the inhibition of basal HCO$_3$/$H_1$ absorption by both agents is stable and reversible and is similar in magnitude to that induced by amiloride and NGF. Second, jasplakinolide and latrunculin B inhibit HCO$_3$/$H_1$ absorption with different time courses, but for each agent the time course for inhibition of HCO$_3$/$H_1$ absorption parallels its effect on the cytoskeleton. The different time courses argue against an effect of these agents to bind and directly inhibit NHE1, consistent with previous results (25). Third, jasplakinolide has no effect on HCO$_3$/$H_1$ absorption under two conditions.
different conditions in which basolateral NHE1 is inhibited, arguing against nonspecific effects on ion transport pathways. Fourth, disruption of microtubules does not prevent NHE1-induced transport regulation, indicating that there is specificity in the cytoskeletal signaling pathway for actin filaments. Fifth, the regulation of HCO₃⁻ absorption by AVP and hyposmolality is unaffected by jasplakinolide or latrunculin B, indicating that these agents act selectively to block regulation mediated through NHE1. In the MTAL, AVP decreases HCO₃⁻ absorption by inhibiting apical NHE3 via cAMP (13, 30, 36); hyposmolality increases HCO₃⁻ absorption by stimulating apical NHE3 via phosphatidylinositol 3-kinase (33). Our results suggest that these regulatory pathways are intact in MTAL cells in which the actin cytoskeleton is modified by latrunculin B or jasplakinolide or by amiloride or NGF (Figs. 3 and 6) (19, 20). The normal response to AVP under these conditions is somewhat surprising in view of previous studies indicating that cAMP/protein kinase A-dependent inhibition of NHE3 involves cAMP-induced cytoskeletal reorganization (28) and a NHERF-ezrin signaling complex that links NHE3 to actin filaments (7, 26). It is possible that inhibition of NHE3 by cAMP/protein kinase A in the MTAL occurs independently of cytoskeletal interactions or that the cytoskeletal changes that underlie NHE1-induced regulation of NHE3 do not impair cytoskeletal interactions necessary for inhibition by cAMP. Further work on how cAMP inhibits NHE3 in the MTAL will be required to address this issue.

There is precedent for NHE1-induced control of the actin cytoskeleton in nonepithelial cells. Barber and co-workers have demonstrated in fibroblasts that NHE1 functions as a cytoskeleton anchoring protein and regulates a number of cytoskeleton-dependent processes, including adhesion, motility, and actin filament organization and assembly (6, 21–24). This regulation is mediated in part through ERM proteins, which bind NHE1 and form a structural link between NHE1 and actin filaments (24). An unanswered question involves the relationship between the ion transport and F-actin-anchoring functions of NHE1. Pharmacological inhibition of NHE1 was found to impair actin filament assembly and cell adhesion in response to various stimuli in fibroblasts (21–23). In the MTAL, we found that inhibiting NHE1 activity by two different methods (amiloride and NGF) resulted in cytoskeletal changes involving decreased F-actin. These results suggest that NHE1-induced cytoskeletal remodeling may be related to or dependent upon changes in NHE1 transport activity. On the other hand, NHE1-dependent formation of actin stress fibers and focal adhesion assembly were preserved in fibroblasts expressing an NHE1 mutant that bound F-actin but was devoid of transport activity (24). Also, disruption of the cytoskeleton with cytochalasin B had no effect on NHE1 activity in AP-1 cells (25), further supporting a dissociation between NHE1 transport activity and cytoskeletal organization. It is possible, therefore, that conformational changes in NHE1 related to inhibition of transport rather than changes in ion translocation itself are important for mediating NHE1-induced cytoskeletal changes (21–24). Alternatively, the cytoskeletal anchoring and ion transport functions of NHE1 may act cooperatively to regulate actin-dependent cell processes (42). Further studies to examine the relationship between the cytoskeleton and NHE1 transport activity and to identify accessory proteins that may mediate NHE1-cytoskeleton interactions are needed to address these questions in the MTAL.

NHE1-induced reorganization of the cytoskeleton regulates apical NHE3 activity in the MTAL. A role for the actin cytoskeleton in modulating NHE3 activity has been identified previously. In NHE3-transfected AP-1 cells, either latrunculin B or jasplakinolide decreased NHE3 activity (25), consistent with results of the present study showing that these agents inhibited basal HCO₃⁻ absorption in the MTAL. The cytoskeletal regulation of NHE3 involves a region of its cytoplasmic tail that may interact with NHERF and ezrin (25, 27). However, the mechanism by which the cytoskeleton regulates NHE3 activity remains unclear. In AP-1 cells, cytoskeletal changes inhibit NHE3 through effects on its intrinsic activity with no effect on the number of cell surface transporters, arguing against an effect on NHE3 trafficking (25, 27). In contrast, a role for the cytoskeleton in mediating endothelin-induced exocytotic insertion of NHE3 was suggested in OKP cells (29), and cytochalasin D was found to alter the amount of surface biotinylated NHE3 in cultured mouse kidney cells (43). Thus, the mechanisms involved in cytoskeleton-induced regulation of NHE3 are likely to be complex and may depend on the experimental conditions, physiological stimulus, or cell type. Studies examining NHE3 activity, kinetics, and subcellular localization will be required to understand how NHE1-induced cytoskeletal changes regulate NHE3 in the MTAL. Although inhibition of NHE3 is the primary mechanism responsible for the NHE1-induced decrease in HCO₃⁻ absorption in the MTAL (19, 20), our results do not rule out the possibility that cytoskeleton remodeling also may influence basolateral HCO₃⁻ efflux pathways.

In addition to NHE3, other ion transporters critical for epithelial function are regulated through their interactions with the cytoskeleton. These include the epithelial Na⁺ channel ENaC, the ROMK K⁺ channel, the Na⁺-K⁺-2Cl⁻ cotransporter NKCC2, the cystic fibrosis transmembrane conductance regulator Cl⁻ channel, and the AE1 Cl⁻/HCO₃⁻ exchanger (21, 39, 44–48). NHE1 is expressed along with these transporters in a variety of epithelial tissues, including the renal proximal tubule, thick ascending limb, and collecting duct; the gastrointestinal tract; pancreatic acinar cells; bile duct; and salivary gland. Our results thus raise the possibility that NHE1 acting via the cytoskeleton could influence a broad range of membrane transporters and epithelial functions, as it does NHE3 and HCO₃⁻ absorption in the MTAL. A loss of cross-talk between basolateral NHE1 and apical Na⁺/H⁺ exchange has been suggested recently as a possible explanation for decreased NaCl absorption in the MTAL. A role for the actin cytoskeleton in modulating NHE3 activity has been identified previously.
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absorption by parotid gland cells from NHE1 knockout mice (49). An effect of NHE1 to control NHE3 or other transporters may also be relevant to pathophysiologic conditions in which NHE1 expression and activity are altered. For example, increased NHE1 activity contributes to ischemic-reperfusion injury of myocardial cells, a process that involves regulatory interactions of NHE1 with other transporters such as Na+/Ca2+ exchange (50, 51). Also, NHE1 activity is increased in a variety of cells and tissues in patients with essential hypertension and in hypertensive animal models (52, 53), and transgenic mice overexpressing NHE1 exhibit salt-sensitive hypertension in association with renal Na+ retention (54). Our results suggest that an increase in basolateral NHE1 activity leading to secondary stimulation of apical NHE3 could promote renal Na+ retention and contribute to the pathophysiology of salt-sensitive hypertension.

In summary, our findings indicate that basolateral NHE1 controls the activity of apical NHE3 in the MTAL through actin cytoskeleton remodeling. Inhibition of NHE1 induces a decrease in polymerized actin that decreases apical NHE3 activity and transepithelial HCO3- absorption. Our results suggest that NHE1 could influence a broad range of ion transporters and epithelial transport functions by controlling the organization of the actin cytoskeleton.

Acknowledgments—Confocal microscopy was carried out in the University of Texas Medical Branch Optical Imaging Laboratory. We thank L. Vergara, director of this laboratory, for important advice on the imaging experiments. We also thank G. Shull and L. Reuss for critical reading of the manuscript.

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J. Biol. Chem. 2005, 280:11439-11447.
doi: 10.1074/jbc.M410719200 originally published online January 11, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M410719200

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