Communication

Activation of Na\(^+-\)H\(^+\) Exchange Is Necessary for RhoA-induced Stress Fiber Formation*

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The ubiquitously expressed Na\(^+-\)H\(^+\) exchanger isoform, NHE1, functions in regulating intracellular pH and cell volume. We recently determined that the GTPase G\(_{a13}\) stimulates NHE1 activity through a RhoA-dependent mechanism (Hooley, R., Yu, C.-Y., Symons, M., and Barber, D. L. (1996) J. Biol. Chem. 271, 6152–6158). RhoA belongs to the Ras superfamily of GTPases and is a key regulator of actin stress fiber formation. We therefore investigated the relationship between RhoA, NHE1 activity, and the regulation of stress fiber assembly. Using two independent approaches, pharmacological inhibition of NHE1 and NHE1-deficient cells, we determined that the induction of stress fibers by lysophosphatidic acid and RhoA is dependent on increased NHE1 activity. These results indicate that stimulation of NHE1 acts downstream of RhoA in a pathway that controls stress fiber formation.

The members of the Rho family of low molecular weight GTP-binding proteins are key control elements of the organization of the actin cytoskeleton (1). RhoA regulates the formation of stress fibers (2, 3), Rac controls the dynamics of lamellipodia (2–4), and Cdc42 controls filopodia (3). Recently, it has been shown that these GTPases also regulate a wide range of other cell functions, including cell-cell and cell-substrate adhesion, cell proliferation, and lipid metabolism (1). The signaling pathways which control these various functions are still largely uncharted.

RhoA has been shown to regulate the formation of stress fibers via at least two independent pathways. One pathway controls the establishment of focal complexes and is mediated by a staurosporine-inhibitable kinase. Another pathway regulates the polymerization of actin (3). Multiple RhoA effector proteins have recently been identified, including several kinases which could be involved in the regulation of the diverse aspects of stress fiber formation. These include the serine/threonine kinases protein kinase N (5, 6), RhoA-activated kinase (6–8), and PI 5-kinase\(^1\) (9). We recently determined that RhoA also mediates activation of the Na\(^+-\)H\(^+\) exchanger isoform NHE1 by the GTPase G\(_{a13}\) (4). Expression of a constitutively active RhoAV14 stimulates NHE1 activity, and activation of the exchanger by constitutively active a13Q226L is inhibited by coexpression of a dominant interfering RhoAN19. NHE1 is ubiquitously expressed and plays a central role in intracellular pH (pH\(_i\)) homeostasis and cell volume regulation. Increases in NHE1 activity are correlated with increased cell proliferation (10), differentiation (11, 12), and neoplastic transformation (13, 14).

The ability of RhoA to function as an important control point in both the activation of NHE1 and the formation of stress fibers prompted us to investigate the role of NHE1 in the regulation of stress fibers. Using a selective inhibitor of NHE1 and a cell line which is NHE-deficient, we determined that stress fiber formation induced by lysophosphatidic acid (LPA) is dependent on NHE1 activity. Moreover, induction of stress fibers by expression of the constitutively active mutant RhoAV14 is abolished in the NHE1-deficient cell line, but restored after the stable expression of NHE1 in these cells. These results indicate that stimulation of NHE1 activity is an essential component of the signal transduction pathway involved in the regulation of stress fibers by RhoA.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—CCL39 cells, a hamster lung fibroblast line which expresses only the NHE1 isoform; PS120 cells, an NHE-deficient clone derived from CCL39 cells (15); and PS120 cells stably expressing NHE1 (PS120-NHE1) were maintained in high glucose (4.5 g/liter) Dulbecco's modified Eagle's medium (DME), supplemented with 5% heat-inactivated fetal bovine serum (Life Technologies, Inc.), streptomycin (100 \(\mu\)g/ml), and penicillin (100 units/ml). PS120-NHE1 cells were obtained by stable transfection of PS120 cells with the complete coding region of the rat NHE1 cDNA (provided by Dr. J. Lowsky, McGill University) using the LipofectAMINE reagent according to the manufacturer's specifications (Life Technologies, Inc.). After transfection, cells were maintained in DME supplemented with 5% fetal bovine serum for 2–3 days. Cells expressing NHE1 were selected by a proton suicide technique developed by Pouyssegur and colleagues (15). One day after transfection, cells were washed twice with a HEPES buffer and incubated for 60 min in a modified HEPES buffer supplemented with 50 mM NH\(_4\)Cl at 37 °C in a CO\(_2\)-free atmosphere. After washing, the cells were incubated for an additional 2 h in a HEPES buffer (without NH\(_4\)Cl) at 37 °C in a CO\(_2\)-free atmosphere, washed, and maintained in DME as described above. This acid selection was repeated 3 times over a 2-week period. Expression of NHE1 was confirmed by immunoprecipitating NHE1 from \(3^\text{5S}\)-labeled PS120-NHE1 cells and by measuring pH recovery from an acid load in a HEPES buffer (4).

For transient expressions, cells were plated on glass coverslips at 0.5 × 10\(^6\) cells per 60-mm dish 18 h prior to transfection. Cells were transfected using LipofectAMINE and 3 \(\mu\)g of mutationally activated Myc-RhoAV14 or Myc-RacV12. After 24 h, the cells were transferred to serum-free DME containing 2 g/liter NaHCO\(_3\) and maintained for an additional 24 h before experiments. pEXV-MycRacV12 (16) and pEXV-MycRhoAV14 (17) were described previously.

NHE1 Activity and Intracellular pH—NHE1 activity and pH were determined using the fluorescent pH-sensitive dye BCECF (18). Cells plated on glass coverslips were loaded with 1 \(\mu\)M BCECF for 10 min at 37 °C, mounted in a cuvette, and then placed in the thermostat.

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\(1\) The abbreviations used are: PI 5-kinase, phosphatidylinositol 4-phosphate 5-kinase; PBS, phosphate-buffered saline; DME, Dulbecco's modified Eagle's medium; BCECF, 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein; LPA, lysophosphatidic acid; NHE1, ubiquitous Na\(^+-\)H\(^+\) exchanger; pH\(_i\), intracellular pH.
NHE1 activity. Addition of LPA to HOE694-treated cells caused a slight increase in stress fiber formation compared with quiescent CCL39 cells (data not shown), stress fiber formation induced by LPA was greatly reduced in the presence of HOE694 compared to the absence of this inhibitor. This finding indicates that pharmacological inhibition of NHE1 attenuates LPA-induced stress fiber formation.

We next confirmed that LPA and HOE694 were regulating NHE1 activity. This was determined by measuring the rate of pH$_i$ recovery from an acute acid load induced by 20 mM NH$_4$Cl (4). NHE1 activity was studied in a HEPES buffer to isolate the activities of the Na-H exchanger as well as anion exchangers. The rate of pH$_i$ recovery in quiescent CCL39 cells was significantly increased in the presence of LPA (Fig. 1D; p < 0.01), indicating that LPA stimulated NHE1 activity. Pretreatment of CCL39 cells with HOE694 completely eliminated the pH$_i$ recovery, indicating that in a HEPES buffer, H$^+$ efflux after an acid load was mainly due to the activity of NHE1. Addition of LPA was unable to rescue the pH$_i$ recovery following HOE694 pretreatment (data not shown). In a HEPES buffer, LPA also induced an increase in steady-state pH$_i$ compared to unstimulated cells (Fig. 1E). Addition of HOE694 to quiescent CCL39 cells decreased the steady-state pH$_i$ and prevented the LPA-induced increase in pH$_i$ (Fig. 1E). To confirm that LPA stimulated NHE1 activity under the conditions used for studying stress fiber formation, we also determined the steady-state pH$_i$ of CCL39 cells in a HCO$_3^-$-containing buffer. In the presence of HCO$_3^-$, acute changes in pH$_i$ are due to the activities of the Na$^+$-H$^+$ exchanger as well as anion exchangers. The quiescent steady-state pH$_i$ was higher in a HCO$_3^-$-containing buffer than in a HEPES buffer (Fig. 1E). Addition of LPA resulted in a further increase in pH$_i$ of approximately 0.2 unit, which was similar to the ∆pH$_i$ induced by LPA in a HEPES buffer (Fig. 1E). HOE694 alone caused a marked decrease in pH$_i$, indicating the contribution of NHE1 activity to steady-state pH$_i$ relative to the activities of Cl-HCO$_3^-$ exchangers. Addition of LPA to HOE694-treated cells caused an increase in steady-state pH$_i$ to a value similar to that of quiescent, untreated cells. This suggested that LPA might stimulate activity of an acid exchanger, such as the Na$^+$-dependent Cl-
HCO$_3^-$ exchanger, or inhibit activity of an acid loader, such as the Cl-HCO$_3^-$ exchanger, independently of its effect on NHE1. The LPA-induced increase in pH$_i$ in the presence of HOE694 ($\Delta$pH$_i$ 0.08), however, was significantly less than that observed in its absence ($p$, 0.01). These findings indicate that in a HCO$_3^-$-containing buffer, LPA induces an increase in steady-state pH$_i$ that is primarily due to the stimulation of NHE1 and to a lesser extent to the modulation of additional H$_1$-regulating mechanisms.

To further examine the role of NHE1 in stress fiber formation, we used PS120 fibroblasts, an NHE-deficient cell line derived from CCL39 cells (15). The morphology of PS120 cells differed from that of parental CCL39 cells in that their shape was more fusiform and their cytoplasm contained only diffusely distributed actin filaments. In quiescent PS120 cells, no stress fibers were apparent (Fig. 2A). In contrast to CCL39 cells, LPA failed to induce stress fiber formation in PS120 cells (Fig. 2B). Stable expression of NHE1 (PS120-NHE1), however, rescued the ability of LPA to induce stress fibers (Fig. 2D), indicating a requirement for the exchanger in this process. PS120-NHE1 cells also had a cell shape that was similar to that of parental CCL39 cells. Western analysis with anti-actin antibodies indicated that the abundance of actin was similar in CCL39, PS120, and PS120-NHE1 cells (data not shown), suggesting that the lack of stress fiber formation in PS120 cells was not due to a down-regulation of actin. Although PS120 cells do not express Na$^+$-H$^+$ exchangers, their quiescent steady-state pH$_i$ in a HCO$_3^-$-containing buffer was higher than that of HOE694-treated CCL39 cells, perhaps due to a compensatory regulation of Cl-HCO$_3^-$ exchangers. Addition of LPA resulted in only a small increase in steady-state pH$_i$ (less than 0.06 unit). In contrast, in PS120-NHE1 cells, LPA induced an increase in steady-state pH$_i$ similar to that of CCL39 cells (compare Fig. 1E and Fig. 2D).

To determine whether RhoA-induced stress fiber formation depends on activation of NHE1, we transiently expressed a Myc-tagged, constitutively active RhoAV14 allele in CCL39, PS120, and PS120-NHE1 cells. Cytoskeletal changes were visualized by staining with rhodamine-conjugated phalloidin (A, C, E, and G), and cells expressing Myc-tagged proteins were identified using co-immunostaining with c-Myc polyclonal IgG and fluorescein isothiocyanate fluorescence (B, D, F, and H). (Bar = 15 μm.)

FIG. 2. LPA regulation of stress fiber formation and steady-state pH$_i$ in PS120 and PS120-NHE1 fibroblasts. A–D, stress fiber formation in PS120 cells in the absence (A) and presence of LPA (500 nM) (B) and in PS120-NHE1 cells in the absence (C) and presence of LPA (D). (Bar = 15 μm.) E, steady-state pH$_i$ in PS120 and PS120-NHE1 cells was determined in a HCO$_3^-$-containing buffer in the absence or presence of LPA. Data represent the means ± S.E. of 3–5 determinations.

FIG. 3. Activation of NHE1 is necessary for stress fiber formation. Fluorescence micrographs of Myc-RhoAV14 expression in CCL39 (A and B), PS120 (C and D), and PS120-NHE1 cells (E and F), and of Myc-Rac1V12 expression (G and H) in CCL39 cells. Cytoskeletal changes were visualized by staining with rhodamine-conjugated phalloidin (A, C, E, and G), and cells expressing Myc-tagged proteins were identified using co-immunostaining with c-Myc polyclonal IgG and fluorescein isothiocyanate fluorescence (B, D, F, and H). (Bar = 15 μm.)

To determine whether RhoA-induced stress fiber formation depends on activation of NHE1, we transiently expressed a Myc-tagged, constitutively active RhoAV14 allele in CCL39, PS120, and PS120-NHE1 cells. In CCL39 cells, RhoAV14-expressing cells demonstrated a strong increase in stress fibers and the cells appeared to be contracted (Fig. 3, A and B). In contrast, in PS120-NHE1 cells, LPA induced an increase in steady-state pH$_i$ similar to that of CCL39 cells (compare Fig. 3, E and F). Together, these experiments indicate that NHE1 is critical for RhoA-mediated formation of stress fibers.
In order to determine whether NHE1-dependent cytoskeletal changes were specific to those induced by RhoA, we also examined the role of NHE1 in lamellipodia formation induced by a constitutively active RacV12. We previously demonstrated that although expression of RacV12 stimulates NHE1 activity, it acts through a mitogen-activated protein kinase kinase 1 (MEKK1)-dependent mechanism that is independent of RhoA (4). Expression of RacV12 in PS120 cells caused extensive lamellipodia formation and membrane ruffling (Fig. 3, G and H), similar to the Rac1-induced phenotype previously reported in CCL39 cells (4) and other cell types (3, 22). This suggested that lamellipodia formation by Rac1 is independent of NHE1 activity.

We have presented two lines of evidence suggesting that NHE1 activity is critical for RhoA-mediated assembly of stress fibers. First, pharmacological inhibition of the exchanger with HOE694 inhibited LPA-induced stimulation of NHE1 activity and stress fiber formation. Second, although the cytoskeletal effects of LPA and RhoAV14 were absent in NHE1-deficient PS120 cells, they were restored by the stable expression of NHE1. Together, these findings strongly suggest that stimulation of NHE1 by RhoA cooperates with an undetermined RhoA target to induce the formation of stress fibers. Indeed, the establishment of stress fibers is likely to be a complex process involving at least two independent activities: actin polymerization and the assembly or regulating focal adhesions (3). Whether NHE1 plays a role in actin polymerization or focal adhesion assembly remains to be determined. It is interesting to note in this respect that adhesion of anchorage-dependent cells to fibronectin activates Na\(^+\)-H\(^+\) exchange and increases pH\(_i\) in a HCO\(_3\) buffer (25), and NHE1 has been found to be predominantly localized at focal complexes (26).

The exact nature of the involvement of NHE1 in stress fiber formation is still unclear. In Dictyostelium, changes in pH\(_i\) have been shown to regulate the organization of the actin cytoskeleton by altering the bundling activity of EF1α (27). Increases in pH\(_i\), however, are associated with a decrease in actin bundling by EF1α. Additionally, other actin-binding proteins, including hisaactofilin (28), α-actinin (29), and talin (30), show reduced actin binding with increasing pH\(_i\). It seems unlikely, therefore, that a direct effect of pH\(_i\), on the properties of actin-binding proteins could account for the role of NHE1 in stress fiber formation. Alternatively, there may be events other than pH\(_i\) that are regulated by NHE1 activity which are critical for stress fiber assembly, such as the concentration of intracellular Na\(^+\) or an as yet unidentified signal produced by NHE1.

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Intracellular distribution of Arf proteins in mammalian cells. Arf6 is uniquely localized to the plasma membrane.

Margaret M. Cavenagh, J. Andrew Whitney, Kathleen Carroll, Chun-jiang Zhang, Annette L. Boman, Anne G. Rosenwald, Ira Mellman, and Richard A. Kahn

Page 21772, Fig. 9: The incorrect figure was printed. The correct figure is shown below:

![Image of immunoblots](image)

**FIG. 9.** Arf6 is not present on brain or liver clathrin-coated vesicles. Immunoblots, performed as described under “Materials and Methods,” were developed with either 1D9 (pan-Arf) or R-1471 (anti-Arf6) antibodies are shown. Positive controls included purified, recombinant Arf1 (lane 1 = 10 ng; lane 2 = 30 ng) and Arf6 (lanes 5 and 8 = 20 ng). Lanes 3 and 6 are 10 μg of bovine brain, and lanes 4 and 7 are 10 μg of rabbit liver clathrin-coated vesicle proteins. Lanes 7 and 8 were intentionally overexposed to increase sensitivity. The data shown are a composite from two different experiments, each of which has been repeated at least twice with the same result. Note the absence of Arf6 immunoreactivity in both brain and liver coated vesicle preparations.

Page 21773: Footnote 5 should be deleted.

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Page 22283, Fig. 3 legend, line 4: This line should read “PS120 cells,” not “CCL39 cells.” The correct legend is shown below.

**Fig. 3. Activation of NHE1 is necessary for stress fiber formation.** Fluorescence micrographs of Myc-RhoAV14 expression in CCL39 (A and B), PS120 (C and D), and PS120-NHE1 cells (E and F), and of Myc-Rac1V12 expression (G and H) in PS120 cells. Cytoskeletal changes were visualized by staining with rhodamine-conjugated phalloidin (A, C, E, and G), and cells expressing Myc-tagged proteins were identified using co-immunostaining with c-Myc polyclonal IgG and fluorescein isothiocyanate fluorescence (B, D, F, and H). (Bar = 15 μm).

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