Role of the Cytoskeleton in Mediating cAMP-dependent Protein Kinase Inhibition of the Epithelial Na\(^+\)/H\(^+\) Exchanger NHE3*

Katalin Szász†§, Kazuyoshi Kurashima‡, Kozo Kaibuchi§, Sergio Grinstein¶, and John Orlowski**†††

From the †Department of Cell Biology Programme, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada, the §Division of Signal Transduction, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0101, Japan, and the **Department of Physiology, McGill University, Montreal, Quebec H3G 1Y6, Canada

The Na\(^+\)/H\(^+\) exchanger NHE3 isoform mediates the entry of Na\(^+\) into epithelial cells of the kidney and gastrointestinal tract. Hormones and pharmacological agents that activate cAMP-dependent protein kinase A (PKA) are potent inhibitors of native and ectopically expressed NHE3 in epithelial and Chinese hamster ovary AP-1 cells, respectively. Previous studies have shown that acute inhibition is coupled to direct phosphorylation of the exchanger, but this only partly accounts for the observed effects. In this report, we show that inhibition of NHE3 activity by forskolin, an activator of adenylate cyclase, occurs without changes in surface expression of the exchanger but is associated with altered cytoskeletal structure. This effect resembles that obtained with cytochalasin D or latrunculin B, actin disrupting agents that also inhibit NHE3. Such similarities prompted us to further investigate the relationship between PKA-induced inhibition of the exchanger and changes in the actin cytoskeleton. Inhibition of NHE3 by cytochalasin D does not require PKA, because the inhibitory effect is preserved in a mutant NHE3 that is not phosphorylated by PKA and in cells pretreated with the PKA inhibitor H89. In contrast, involvement of actin in the effect of cAMP on the exchanger is supported by the following observations: (i) jasplakinolide, an F-actin stabilizer, prevents the inhibition caused by forskolin, and (ii) constitutively active forms of RhoA and Rho kinase interfere with actin disruption by forskolin and also decrease inhibition of the transporter. These results suggest that reorganization of the cytoskeleton by PKA is involved in mediating inhibition of NHE3.

Members of the Na\(^+\)/H\(^+\) exchanger (NHE)\(^1\) gene family mediate the electroneutral exchange of Na\(^+\) for H\(^+\) across biological membranes (see Refs. 1–3 for reviews). To date, seven NHE isoforms have been described in mammals. Three of these are expressed ubiquitously, whereas the others show a more restricted pattern of expression. NHE1 is present in the plasma membrane of most cells, where it functions in the regulation of cytosolic pH and cellular volume. NHE6 and NHE7 are also ubiquitously expressed but accumulate preferentially in mitochondrial and trans-Golgi network/endoosomal membranes, respectively, likely contributing to the ionic homeostasis of these organelles (4, 5). By contrast, NHE2–5 are expressed only in selected tissues, suggesting more specialized functions. Of these isoforms, the most is known about NHE3, which is localized exclusively in the apical membrane of epithelial cells of the kidney and gastrointestinal tract (6, 7). By catalyzing the entry of Na\(^+\) into these cells, NHE3 has a central role in maintaining systemic electrolyte and fluid balance.

A large variety of factors, including several hormones (2, 8) and physical parameters such as osmolarity (9, 10), influence NHE3 function, contributing to the fine control of electrolyte homeostasis. Hormones that elevate intracellular cAMP levels are potent inhibitors of NHE3, an effect that is partly attributed to direct phosphorylation of the exchanger by protein kinase A (PKA). Mutation of Ser\(^{605}\), the prime target of PKA in NHE3, reduces the acute inhibitory effect of cAMP by only \(\sim50\%)\ (12). This finding points to the existence of other modes of action of cAMP that indirectly regulate NHE3 activity.

Recent evidence has shown that, in addition to being present at the surface membrane, NHE3 is also detectable in intracellular vesicles (14). In epithelial cells, where it is expressed endogenously, the presence of NHE3 in endomembrane vesicles has been documented by both electron microscopy and confocal immunofluorescence microscopy (15, 16). This distribution pattern is recapitulated when NHE3 is heterologously expressed in Chinese hamster ovary cells (17). In these cells, plasmalemmal transporters undergo constitutive uptake into clathrin-coated vesicles (18) and are recycled back to the plasma membrane in a phosphatidylinositol 3-kinase-dependent manner. Inhibition of this kinase with wortmannin or LY294002 blocks the exocytosis of vesicles bearing NHE3, depleting the plasmalemmal pool of exchangers and causing a marked drop in the transport rate (19, 20). Similarly, acute inhibition of NHE3 activity upon activation of protein kinase C in colonic epithelial cells was attributed to net internalization of exchangers from the brush border into a subapical cytoplasmic compartment (16).

* This work was supported by the Canadian Institutes of Health Research and the Kidney Foundation of Canada. 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.

§ Supported by a Canadian Institutes of Health Research fellowship. ¶ International Scholar of the Howard Hughes Medical Institute. Current holder of the Pritzlak Chair in Cell Biology at The Hospital for Sick Children. Cross-appointed to the Department of Biochemistry, University of Toronto. To whom correspondence should be addressed: Cell Biology Program, Hospital for Sick Children, 555 University Ave., Toronto, ON M5G1X8, Canada. Tel.: 416-813-5727; Fax: 416-813-5028; E-mail: sga@sickkids.on.ca.

** Investigator of the Canadian Institutes of Health Research.

††† The abbreviations used are: NHE, Na\(^+\)/H\(^+\) exchanger; BCECF, 2’/7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; eGFP, enhanced green fluorescent protein; HA, hemagglutinin; MLC, myosin light chain; NHERF/EBP50, NHE regulatory factor/ezrin-binding protein of 50 kDa; PBS, phosphate-buffered saline; pHi, cytosolic pH; PKA, protein kinase A; ROY, p160 Rho-associated kinase I
The preceding results indicate that modulation of the dynamic equilibrium between surface and endomembrane transporters can provide a rapid and effective means of controlling the rate of ion exchange. This mechanism could, in principle, also mediate the inhibitory effects of cAMP.

Indeed, upon exposure of renal cells to parathyroid hormone, which elevates cAMP, the number of NHE3 transporters detectable at the cell surface decreased (21). However, the acute inhibition of transport did not parallel the course of disappearance of the exchangers, which occurred over more prolonged treatment, implying that other processes must be involved in the effect of cAMP.

Because neither direct phosphorylation nor modulation of traffic fully account for the acute cAMP-induced inhibition of NHE3, a role for ancillary proteins has also been invoked (22). Proteins containing PDZ domains, including NHERF1/EBP50 (23) and NHERF2/TKA-1/E3KARP (24), were shown to bind directly to NHE3 and postulate to mediate the effect of PKA. NHERF1/EBP50 also binds to ezrin, which is thought to bridge membrane proteins with the actin cytoskeleton. Because ezrin is a PKA-anchoring protein, it may function to recruit the kinase to the vicinity of the NHERF/NHE3 complex, promoting the phosphorylation of the exchanger and/or cytoskeletal proteins. The latter have also been implicated in the control of NHE3 (25). Disruption of normal actin structure by pharmacological means or by an inactive RhoA construct impaired NHE3 function without altering the number of NHE3 molecules at the plasma membrane (26, 27).

The inhibition of NHE3 by cytoskeleton-disrupting agents bears some similarities with that induced by elevation of cAMP. Moreover, activation of PKA has been reported to alter the structure of the actin cytoskeleton (28–31). These similarities prompted us to consider the possibility that cAMP inhibits NHE3, at least in part, by modifying the cytoskeletal architecture. In this report, we analyzed the effects of forskolin, an activator of adenylyl cyclase, on the control of actin via RhoA and assessed the relationship between these changes and the activity of NHE3. To facilitate the functional analysis of NHE3 activity, we used cells devoid of endogenous NHE activity that were stably transfected with an epitope-tagged form of NHE3. By placing the epitope tag in an extracellular loop of NHE3, we were able to monitor the distribution and traffic of the exchangers before and after elevation of cAMP.

EXPERIMENTAL PROCEDURES

Materials and Solutions—Nigericin, the acetoxyethyl ester of 2,7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BECFCF), jasplakinolide, Oregon green-labeled phalloidin, and rhodamine-phalloidin were from Molecular Probes, Inc. (Eugene, OR). H9 medium (NaCl, 60 mm KCl, 2 mm MgCl2, and 2 mm CaCl2, 20 mm Hepes, pH 7.4) was from Roche Molecular Biochemicals. 125I-Labeled goat anti-mouse IgG was from ICN Pharmaceuticals. Materials and Solutions—

Measurement of Na+/H+ Exchanger Activity—NHE3 activity was assayed as the rate of Na+-induced recovery of cytosolic pH (pHi) following an acid load, imposed by prepulsing with NH4Cl, as described previously (9). Briefly, the cells grown on coverslips to ~70% confluence were incubated with 2 μM of the acetoxyethyl ester precursor of BECFM plus 50 mM NH4Cl at 37 °C. After 10 min, the cells were placed into Leiden coverslip holders and washed with isotonic Na+-free solution to remove excess dye and extracellular NH4Cl. Na+/H+ exchange was then initiated by reintroduction of extracellular Na+ and was estimated from the rate of recovery of pHi. Two methods were used to measure the fluorescence of BECFM. Population fluorescence was performed using a Nikon Diaphot TMD inverted microscope coupled to the M Series dual wavelength illumination and recording system from Photon Technologies, Inc. (South Brunswick, NJ) in a dual excitation, single emission configuration, as detailed earlier (9).

The fluorescence of transiently transfected single cells was measured using a confocal two-photon imaging system as described in Ref. 38 controlled by the Metafluor software (Universal Imaging, West Chester, PA). Transfected cells were identified by detecting eGFP fluorescence prior to loading with BECFM. A neutral density filter was then interposed in the excitation pathway to decrease the signal emanating from eGFP, and the cells were loaded with BECFM and ammonium while on the microscope stage. The fluorescence of BECFM greatly exceeded that of eGFP allowing it to be readily visible in the presence of the neutral density filter. Where specified, the cells were pretreated with 10 μm forskolin for 10 or 30 min. Similar results were obtained at both times.

The excitation wavelengths were 440 and 490 nm, and the emission wavelength was 510 nm for both the population and single cell measurements. In both instances the pH was calibrated by equilibrating the cells with a K+-rich medium titrated to known pH values and containing 10 μg/ml nigerin, as described previously (39).

Immunofluorescence—AP-1 cells stably expressing NHE333HA were plated onto glass coverslips and grown to ~70% confluence. To visualize the total cellular NHE333HA, the cells were fixed for 15 min at room temperature using 4% paraformaldehyde in PBS and were then permeabilized with 0.1% Triton X-100 in PBS for 20 min at room temperature, blocked with 5% milk in PBS for 1 h, and incubated with mouse anti-HA antibody for 1 h. The coverslips were next washed four or five times with PBS and incubated with Cy3-conjugated anti-mouse IgG for 1 h. After incubation with the secondary antibody, the cells were washed three to five times over 15 min with PBS and were mounted onto glass slides with DAKO fluorescence mounting medium (DAKO Corp., Carpinteria, CA). Where specified, F-actin was labeled by incubating fixed and permeabilized AP-1 cells with Oregon green-labeled phalloidin or rhodamine-phalloidin (1:500) for 1 h at room temperature.

Quantitation of Surface NHE3—Two different methods were used to quantify surface NHE3 expression: (i) a modified enzyme-linked immunosorbent assay described earlier (19) and (ii) a radioisotopic method. AP-1 cells stably expressing NHE333HA were plated onto 12-well plates and grown to ~70% confluence. For enzyme-linked immunosor-
bent assay, the cells were incubated with anti-HA antibody (1:1000 dilution) for 1 h at 4 °C to prevent endocytosis. After washing the cells six times with PBS/a-minimal essential medium (9:1 v/v) to remove excess unbound antibody, they were fixed for 10 min at room temperature using 4% paraformaldehyde in PBS. Following fixation, the cells were washed three or four times with PBS and incubated with 100 mM glycine in PBS for 15 min. The cells were next blocked with 5% donkey serum for 20 min and then incubated with a peroxidase-conjugated donkey anti-mouse antibody (1:1000 dilution) for 1 h followed by washing six more times with PBS/a-minimal essential medium and incubation with 1 μl of o-phenylenediamine dihydrochloride reagent for 15 min at room temperature. The reaction was stopped by adding 250 μl of 3 M HCl. The supernatant was collected and absorbance measured at 492 nm using a U-2000 spectrophotometer (Hitachi). In the range studied, the absorbance varied linearly with the amount of peroxidase bound.

For isotopic determinations, the cells were treated on the plates as specified in the text and then placed on ice. All subsequent steps were carried out at 4 °C to prevent further traffic of NHE3. The cells were next washed three times with PBS and then incubated with 125I-labeled goat anti-mouse IgG (0.4 μCi/sample) in HPMI with 10% goat serum for 45 min. At the end of the incubation the cells were washed three more times with PBS to remove unbound radiolabel and detached from the plates using PBS containing 1% Triton X-100. Radioactivity was counted with a 1282 Compugamma LKB counter. The radioactivity bound to cells exposed only to 125I-IgG without prior incubation with anti-HA antibody was also determined and subtracted from all determinations. The data are expressed as percentages of surface labeling of untreated control cells (100%) and are the means ± S.E. of the number of experiments indicated, each performed in duplicate or triplicate.

Assessment of Myosin Phosphorylation—The cells grown to ~70% confluency in 10-cm dishes were treated as specified in the text. After rinsing with PBS, their proteins were precipitated with ice-cold 10% trichloroacetic acid in acetone containing 2 mM dithiothreitol. The pre-rinsing with PBS, their proteins were precipitated with ice-cold 10% trichloroacetic acid in acetone. The pellets were solubilized in sample buffer containing 9M urea, 2 mM dithiothreitol, 22 mM glycine, and 20 mM Tris, pH 8.8. Equal volumes of the lysates were fractionated by glycerol-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Equal sample loading was confirmed by staining with Ponceau S. The blots were probed with a monoclonal anti-myosin light chain antibody (clone MY-21, 1:200 dilution) followed by a peroxidase-coupled anti-IgM antibody. The blots were visualized using enhanced chemiluminescence.

RESULTS

Effect of cAMP on the Subcellular Distribution of NHE3—NHE3 has been shown to undergo recycling between the plasma membrane and intracellular compartments. Increased endocytosis or reduced exocytosis would alter the number of exchangers at the surface and might account for the inhibition of transport induced by cAMP. To examine the effect of cAMP on the trafficking of exchangers between compartments, we used cells stably transfected with a modified form of NHE3 including three tandem copies of an influenza HA epitope (see also Fig. 2B). These results were validated using an indirect enzyme-linked immunosorbent assay method for detection of the secondary antibodies (19), which also showed no difference between control and cAMP-treated cells (surface expression of NHE3 in forskolin-treated cells was 110 ± 5% of untreated cells (n = 4); data not shown). Therefore, the acute inhibitory effect of cAMP does not involve a reduction in the number of transporters present at the cell surface.

Cytoskeletal Effects of Forskolin—in search for an alternative mechanism of action of cAMP, we noticed that AP-1 cells undergo marked morphological changes upon forskolin treatment (Fig. 1, A and B). Similar effects on morphology had been reported for other cell types (30, 31, 40, 41) and were attributed to remodeling of the actin cytoskeleton. Accordingly, visualization of F-actin using Oregon green-labeled phalloidin shows that control AP-1 cells are spread with a number of long, often parallel stress fibers (Fig. 2A). Upon the addition of forskolin, the cells round up leaving processes behind. The number and length of the stress fibers is markedly reduced (Fig. 2B).

The altered morphology and cytoskeletal organization seen in cells treated with forskolin are somewhat reminiscent of the effects of agents that impair actin assembly (e.g. cytochalasin D) or decrease myosin phosphorylation (e.g. the myosin light chain kinase inhibitor ML-9) (26, 38). Indeed, previous studies in other cell types suggested a decrease in myosin phosphorylation to be the cause of the altered cytoskeletal organization induced by cAMP (41, 42). To establish whether the same mechanism underlies the alterations noted in AP-1 cells, we used urea-glycerol gel electrophoresis and immunoblotting to assess the phosphorylation status of myosin. As shown in Fig. 2C, the majority of myosin is phosphorylated in unstimulated control cells. Treatment with forskolin caused a marked shift from the phosphorylated to the nonphosphorylated form. Myosin dephosphorylation could therefore account for the observed morphological and cytoskeletal changes induced by forskolin in AP-1 cells.

Previous studies have shown that optimal NHE3 activity requires an intact actin cytoskeleton. Disruption of the cytoskeleton by actin- or myosin-modifying agents reduces
cAMP Regulation of Na⁺/H⁺ Exchanger NHE3

Na⁺/H⁺ exchange mediated by NHE3 (27). It seemed conceivable, therefore, that cytoskeletal alterations contribute to the inhibitory effects of cAMP on transport. To test this possibility, we initially compared the effects of cytochalasin D and forskolin on NHE3. The activity of the exchanger was monitored as the rate of Na⁺-induced recovery of cytosolic pH (pHi) following an acid load, imposed by prepulsing with NH₄Cl (see “Experimental Procedures”). Fig. 2D shows that, as reported (26, 43), both drugs depress the transport rate, with cytochalasin having a more profound effect. Simultaneous addition of cytochalasin and forskolin did not cause further reduction in NHE3 activity. Because their effects are similar and are not additive, cAMP and cytochalasin may share a common inhibitory mechanism. However, this is difficult to evaluate because of the nearly complete inhibition exerted by cytochalasin.

Effect of F-actin Stabilization on the Forskolin Inhibition—If destabilization of the actin cytoskeleton plays a role in mediating the effect of cAMP on NHE3, agents known to stabilize F-actin are predicted to interfere with the inhibition of the transporter. To test this notion, we used jasplakinolide, a membrane-permeant agent that binds to F-actin, preventing its depolymerization (44). Jasplakinolide or the less permeable compound phalloidin have been used previously to minimize the cytoskeletal effects of cAMP (45). To validate this approach in AP-1 cells, it was important to assess the effects of jasplakinolide on actin polymerization and on the subsequent effect of cAMP. However, jasplakinolide and phalloidin are known to bind at the same sites on F-actin (44). Indeed, pretreatment with jasplakinolide virtually eliminated the subsequent binding of labeled phalloidin (not shown). Although this serves as an indication of effective uptake of jasplakinolide into the cells, the possible stabilizing effect of this drug in cAMP-treated cells cannot be evaluated using phalloidin derivatives. To overcome this problem, we transiently expressed eGFP-labeled G-actin in the AP-1 cells. This labeled chimera has been shown to be effectively incorporated into F-actin. In otherwise untreated cells, the long, parallel stress fibers seen earlier with Oregon green-phalloidin were apparent, although visualization was made somewhat more difficult by the presence of fluorescent cent cytosolic G-actin (Fig. 3A). Jasplakinolide (1 μM) caused marked accumulation of actin into bright patches mostly in the periphery of the cell. Few stress fibers and very little free cytoplasmic G-actin were present (Fig. 3C). A similar effect of long treatment with jasplakinolide was reported earlier for rat embryonic fibroblasts (46). When forskolin was added, the cells expressing eGFP-actin rounded up but remained attached by fine processes, as did the untransfected cells. As before, the stress fibers were diminished, shortened, and irregularly arranged (Fig. 3B). When added after pretreatment with jasplakinolide, forskolin caused less dramatic changes. Although stress fibers were disrupted, cell rounding and the extension of processes were partly prevented (Fig. 3D). Some of the bright actin patches noted in cells treated with jasplakinolide alone persisted after the addition of forskolin.

The functional consequences of jasplakinolide pretreatment were studied next. As shown in Fig. 3E and consistent with earlier observations (26), jasplakinolide alone caused a substantial decrease in the rate of transport. This seemingly paradoxical inhibition may be explained by the finding that, although an F-actin stabilizer, jasplakinolide nevertheless alters the normal actin distribution of the cells (Fig. 3C). More importantly, subsequent addition of forskolin to jasplakinolide-treated cells had no additional effect on NHE3 activity. Thus, stabilization of F-actin prevented the inhibitory effect of cAMP.

Inhibition of NHE3 by Actin Disruption Is Not Mediated by PKA—The above results raise the possibility that the inhibitory effects of cAMP and cytoskeletal disruption on NHE3 share a common mechanism of action. We considered two alternative mechanisms. First, the inhibition by actin disruption could result from subsequent activation of PKA and/or of exposure of PKA phosphorylation sites on the exchanger or an ancillary protein(s). Alternatively, the inhibitory effects of cAMP may be mediated by modification of the cytoskeleton.

To test the first possibility, we investigated the role of NHE3 phosphorylation in the inhibition by actin disruption. We had described previously that Ser605 of NHE338(HA3) becomes phosphorylated in AP-1 cells upon cAMP activation, whereas another neighboring serine residue, Ser634, that is also critical for

---

**FIG. 2. Effect of forskolin on actin structure, MLC phosphorylation, and NHE3 activity.** A and B, AP-1 cells expressing NHE338(HA3) were incubated without (A) or with (B) 10 μM forskolin for 10 min at 37 °C. The cells were then fixed, permeabilized, and stained with Oregon green-phalloidin to visualize F-actin. C, phosphorylation of MLC. The cells were untreated (control) or treated with 10 μM forskolin for the indicated period. After treatment, they were extracted with trichloroacetic acid in acetone, and the precipitate was dissolved in a urea-containing sample buffer. Equal volumes were subjected to urea-polyacrylamide gel electrophoresis, followed by blotting onto nitrocellulose. The blots were probed with anti-MLC antibodies (1:200) and visualized with horseradish peroxidase-conjugated anti-mouse IgM and ECL. The locations of MLC and of its phosphorylated form (P-MLC) are indicated. D, cells expressing NHE3 were grown on coverslips to ~70% confluence. The cells were incubated for 30 min with 10 μM forskolin (triangles), with 10 μM cytochalasin D (solid circles), with both drugs (solid squares), or without additions (control, open circles). In the last 10 min of the treatment the cells were loaded with BCECF-AM and 50 mM NH₄Cl. The cells were then washed with Na⁺-free medium, and their pH was monitored fluorimetrically, as described under “Experimental Procedures.” Recording was initiated upon reintroduction of extracellular Na⁺ to induce Na⁺/H⁺ exchange. The results are the means ± S.E. of nine determinations from three separate experiments.
confering partial responsiveness to cAMP, was not phosphorylated (12). We therefore used mutant forms of NHE3 where these Ser residues were replaced by glycine or alanine. NHE3/S605G is no longer phosphorylated upon forskolin treatment and has a reduced sensitivity to inhibition by forskolin (Ref. 12 and Fig. 4A). Of note, cytochalasin D is still capable of inducing a profound inhibition in cells expressing this mutant (Fig. 4A), implying that phosphorylation of Ser605 is not required for modulation of NHE3 by cytochalasin. As before, forskolin had no detectable additional effect in NHE3/S605G cells treated with cytochalasin (not shown). Likewise, the single mutant S634A responded effectively to cytochalasin (Fig. 4B). By contrast, a dual mutant (NHE3/S605G,S634A), which was shown to be completely unresponsive to cAMP (see inset in Fig. 4C and Ref. 12), was equally insensitive to cytochalasin (Fig. 4C).

Two explanations can be offered: (i) either one of the serines, acting through different mechanisms suffices to support the cytoskeletal effect, whereas both are required for the full effect of cAMP or (ii) the dual mutation induces a conformational change in NHE3 that precludes both the cAMP and the cytoskeletal inhibition. The latter hypothesis implies that direct phosphorylation of NHE3 may not be required for the cytoskeletal effect.

To differentiate between these hypotheses and to test the involvement of Ser/Thr kinases on the inhibitory effect of cytoskeletal modifiers, we used the inhibitor H89. This drug is a potent Ser/Thr kinase antagonist that is particularly effective against PKA (47). When added by itself, 10 μM H89 had little effect on NHE3 activity (not shown). As expected, the kinase inhibitor antagonized the effects of forskolin, confirming mediation by PKA (Fig. 5). However, the inhibitory effect of cytochalasin persisted in the presence of H89 (Fig. 5). This indicates that neither PKA nor any other H89-sensitive kinases are required for modulation of NHE3 by disruption of the cytoskeleton with cytochalasins.

The preceding observations imply that neither activation of PKA nor exposure of PKA phosphorylation sites can account for the inhibitory effects of cytochalasin. Whereas the cytoskeletal effects are seemingly not mediated by PKA-induced phosphorylation, the converse may be true. Namely, the cytoskeleton may be required for at least part of the cAMP response. In agreement with this notion H89, which precluded the inhibitory effect of forskolin on NHE3, also prevented its effect on cytoskeletal rearrangement (data not shown). Collectively, these observations prompted us to further analyze the role of the acto-myosin skeleton in the inhibition of NHE3 by cAMP.

Effect of Forskolin on NHE3 in Cells Expressing Constitutively Active RhoA—The mechanism whereby cAMP alters the cytoskeleton is not fully understood. Potential targets for cAMP regulation include the small GTP-binding protein RhoA and its downstream effector Rho kinase, which are important regulators of actin organization and myosin phosphorylation. In AP-1 cells, active RhoA and Rho kinase as well as myosin phosphorylation are required for optimal NHE3 activity (38). Because RhoA can undergo phosphorylation by PKA, leading to uncoupling from its effectors (48), we investigated whether this GTPase mediates the inhibitory effect of cAMP on NHE3. Cells expressing NHE3/S634A were transiently transfected with the constitutively active form of RhoA (RhoAQ63Lmyc). As anticipated, the active form of RhoA greatly increased the density of stress fibers (Fig. 6, A and B; note that the exposure had to be reduced to illustrate the bright phallolidin fluorescence of transfected cells, making the fibers in untransfected controls comparatively faint). The transfected cells were next exposed to forskolin. Unlike the untransfected controls, which round up and lose stress fibers upon treatment with forskolin, the cells expressing RhoAQ63Lmyc retained their polygonal shape and remained rich in stress fibers (Fig. 6, C and D). Thus, the constitutively active RhoA prevents morphological changes and rearrangement of the cytoskeleton otherwise induced by cAMP. Similar effects were reported earlier (31).

To assess NHE3 function in single transfected cells, we used fluorescence imaging as described earlier (38). NHE3/S634A cells were transiently cotransfected with RhoAQ63Lmyc and eGFP. Under the conditions used for transfection (5:1 ratio of eGFP and RhoAQ63Lmyc vectors), >85% of the transfected cells express both vectors (not shown), thereby validating the use of eGFP as a marker of RhoA expression. Enhanced eGFP-positive cells were identified, followed by loading with BCECF and acid loading using the ammonium prepulse technique. Recovery of pH was induced by readdition of Na⁺. As shown in Fig. 6E, cells transfected with only eGFP (control) showed a fast recovery from the acid load, which was markedly inhibited by forskolin (Fig. 6E). Expression of active RhoA slightly stimulated the recovery (Fig. 6E). Although forskolin still inhibited the recovery in RhoAQ63Lmyc transfectants, the magnitude of the inhibition was significantly lower (70% versus 46% inhibition of the rate of recovery over the first 60 s in control versus RhoAQ63Lmyc transfectants). Thus, constitutively active RhoA partially decreases the effect of forskolin on NHE3, consistent with the notion that PKA acts on NHE3, in part, by altering the cytoskeleton.

Effect of Forskolin on NHE3 in Constitutively Active Rho Kinase-expressing Cells—The modulation of NHE3 by RhoA has been shown to be exerted by one of its downstream effectors, p160 Rho-associated kinase I, also known as Rho kinase or ROCK (38). To validate the involvement of the RhoA-ROK pathway in the inhibition induced by cAMP, we analyzed the effects
with (solid circles) or with 10 μM forskolin (triangles, A and B in inset in C). During the last 10 min of the pretreatment the cells were stained with BCECF and acid-loaded. pHi was measured as in Fig. 2. pH recovery was induced by the readidation of Na⁺ and was monitored flourometrically. A, cells expressing NHE3/S605G. B, cells expressing NHE3/S634A. C, cells expressing NHE3/S605G,S634A. The inset contains data reproduced from Ref. 12 showing that forskolin does not inhibit the NHE3/S605G,S634A mutant. The data are the means ± S.E. of nine determinations from three separate experiments.

Of forskolin in cells expressing an epitope-tagged, constitutively active form of ROK (ROK-CATmyc) (37). Expression of this construct resulted in the formation of thick actin stress fibers and bright actin patches (Fig. 7, A and B), as described earlier (49). When the transfectants were treated with forskolin, this unique actin pattern persisted, indicating that ROK-CATmyc prevents the effect of CAMP on the cytoskeleton (Fig. 7, C and D), as did active RhoA. By contrast, F-actin stress fibers were diminished in untransfected neighboring cells.

Consistent with earlier observations (38) expression of ROK-CATmyc had no significant effect on the basal activity of NHE3 in AP-1 cells (not shown). However, ROK-CATmyc diminished the inhibitory effect of forskolin on NHE3 activity (Fig. 7E). Although forskolin reduced the rate of transport in the first 60 s in eGFP-transfected controls by 66%, the inhibition in cells expressing ROK-CATmyc was only 38%. Together with the results in Fig. 6, the data obtained with ROK-CATmyc implicate cytoskeletal changes in the regulation of NHE3 by PKA.

FIG. 5. Effect of H89 on NHE3 activity. The cells expressing NHE3/S605G were grown on coverslips to ~70% of confluence and pre-treated for 1 h without (solid symbols) or with 10 μM H89 (open symbols). Next, 10 μM cytochalasin (circles) or 10 μM forskolin (squares) were added for 30 min. During the last 10 min of incubation the cells were stained with BCECF and acid-loaded. pHi was measured as in Fig. 2. The data are the means ± S.E. of nine determinations from three separate experiments.

DISCUSSION

It has recently become apparent that cAMP can exert some of its effects by phosphorylation-independent means. The inhibition of NHE3, however, appears to be primarily mediated by PKA-dependent phosphorylation, because the presence of the kinase antagonist H89 precluded this effect. Therefore, direct phosphorylation of NHE3 or phosphorylation of a distinct regulatory protein may account for the cAMP-induced inhibition, and these alternatives are not mutually exclusive. The first model is supported by earlier findings that Ser⁶⁰⁵ and Ser⁶⁰⁵ of NHE3 become phosphorylated when cAMP is elevated (12, 13). On the other hand, mutation of sites that are not directly phosphorylated (e.g. Ser⁶³⁴) influences the ability of PKA to inhibit NHE3 (12). Such mutations must modify the conformation of the exchanger, altering its ability to interact with regulatory molecules that may, in turn, be substrates of PKA. It is noteworthy that such conformation-sensitive sites reside within a region (residues 585–660) defined to mediate the interaction of the exchanger with NHERF1/2 (50), which in addition contains a potential ezrin binding motif (RKRL, residues 656–659). Because both NHERF/EBP50 and ezrin are purported linkers of membrane proteins with the cytoskeleton and because the latter is also a modulator of NHE3 activity, we explored the possibility that PKA functions in part by remodelling acto-myosin.

We found that elimination of Ser⁶⁰⁵, the direct target of PKA, had little effect on the ability of cytochalasin to inhibit NHE3, suggesting a phosphorylation-independent process. In contrast, a dual mutation at positions 605 and 634 precluded the cytoskeletal effect. Because residue Ser⁶³⁴ is not phosphorylated by PKA, its mutation must have affected the susceptibility of the exchanger to inhibition by conformational means. In support of this notion, we found that the inhibitory effect of cytochalasin is unaffected by the presence of H89, implying a phosphorylation-independent process. These observations serve as a reminder that even conservative mutations can introduce disruptive conformational changes and emphasize that caution must be applied when interpreting mutagenesis studies.

Although the results obtained with H89 rule out the possibility that cytoskeletal disruption by cytochalasin acts via PKA, the converse may still apply. In this context, stimulation of PKA had been reported in several cellular systems to disrupt the normal organization of the actin skeleton (28–31), and a similar effect was also observed in AP-1 cells. The following lines of evidence support the notion that, in addition to directly phosphorylating NHE3, PKA also modulates the exchanger indirectly via the actin cytoskeleton. First, stabilization of F-actin with jasplakinolide blocked the effect of forskolin on NHE3. Secondly, transfection of the constitutively active form of RhoA and its downstream effector Rho kinase reduced the effectiveness of forskolin. Like jasplakinolide, these active mutants stabilize the actin cytoskeleton against the changes otherwise induced by PKA stimulation. The finding that neither forskolin nor cytochalasin altered the number of surface exchangers is also consistent with a common mode of action.

The simplest model to explain the indirect effects of cAMP on NHE3 involves the phosphorylation of RhoA by PKA. In lymphocytes, direct phosphorylation by PKA was found to uncouple RhoA from its effectors, which include Rho kinase (48). The latter controls the state of MLC phosphorylation (51). As shown in Fig. 2, net dephosphorylation of MLC was documented in
AP-1 cells treated with forskolin, which may account for the cytoskeletal reorganization revealed by phalloidin staining. How the cytoskeleton is linked to NHE3 remains obscure, but NHERF1/EBP50, NHERF2/TKA-1/E3KARP, and ezrin are possible candidates. The state of phosphorylation of ezrin, which is itself a substrate of Rho kinase, dictates its ability to associate with transmembrane and cytoskeletal ligands (52). This multiplicity of potential targets may amplify the consequences of RhoA phosphorylation by PKA.

Involvement of the cytoskeleton or RhoA in mediating the effects of cAMP has been invoked earlier for other ion transporters. Stabilization of F-actin using phalloidin prevented cAMP-induced Cl⁻ secretion (53) and activation of the Na/K/Cl cotransporter in T84 cells (54). Also, inactivation of small GTPases of the Rho family using C3 exotoxin attenuated the effects of parathormone on Na-Pi cotransport, which are thought to be mediated by PKA (55). As well, the actin skeleton is seemingly involved in the regulation of Na⁺ and Cl⁻ channels by cAMP (56, 57). The nature of the cytoskeletal interactions and the precise mode of regulation are not known in any of these cases, but it is apparent that modulation of membrane-skeleton association is a widespread and effective means of transport regulation.

In summary, regulation of NHE3 by PKA likely involves more than one mechanism. We suggest that remodeling of the cytoskeleton and inactivation of the RhoA-Rho kinase-myosin pathway synergize with direct phosphorylation of NHE3 at residue Ser605 and indirectly at Ser634 to inhibit cation exchange. Whether the cytoskeleton also participates in NHE3 regulation by other stimuli such as hyperosmolarity, where direct phosphorylation of the transporter is thought not to be involved, remains to be elucidated and is currently under investigation.

REFERENCES
1. Orlowski, J., and Grinstein, S. (1997) J. Biol. Chem. 272, 22373–22376
2. Wakabayashi, S., Shigekawa, M., and Pouyssegur, J. (1997) Physiol. Rev. 77, 51–74
3. Counillon, L., and Pouyssegur, J. (2000) J. Biol. Chem. 275, 1–4
4. Numata, M., Petrecca, K., Lake, N., and Orlowski, J. (1996) J. Biol. Chem. 273, 6951–6959
Role of the Cytoskeleton in Mediating cAMP-dependent Protein Kinase Inhibition of the Epithelial Na⁺/H⁺ Exchanger NHE3
Katalin Szászi, Kazuyoshi Kurashima, Kozo Kaibuchi, Sergio Grinstein and John Orlowski

J. Biol. Chem. 2001, 276:40761-40768.
doi: 10.1074/jbc.M106724200 originally published online August 24, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106724200

Alerts:
• When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 57 references, 32 of which can be accessed free at http://www.jbc.org/content/276/44/40761.full.html#ref-list-1