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Regulation of the Epithelial Na^+/H^+ Exchanger Isoform by the Cytoskeleton

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Key Words

NHE3 • Actin • Na^+ transport • Cell volume regulation • pH regulation

Abstract

Members of the Na^+/H^+ exchanger (NHE) family mediate electroneutral countertransport of H^+ for Na^+ across cellular membranes. The six known isoforms mediate transepithelial Na^+ transport processes and housekeeping functions such as the regulation of cellular and organellar pH and volume. NHE3 is found primarily in the apical membrane of epithelial cells of the kidney and gastrointestinal tract, where it mediates Na^+ (re)absorption. Its fine regulation, whether by hormones that utilize cAMP as a signalling mechanism, or by physical parameters such as the cell volume, provides the adjustments necessary for the maintenance of systemic salt and fluid balance. Although the exact molecular mechanism of this control is unknown, two major modes of regulation have been invoked: 1) alteration of the number of cell surface transporters by changes in the rate of endocytosis and/or exocytosis and 2) regulation of the intrinsic activity of the individual exchangers.

NHE3 requires an intact cytoskeleton for its optimal function. Pharmacological interference with actin polymerization or myosin phosphorylation markedly inhibits the exchanger, without altering the number of transporters exposed at the surface. This effect is isoform specific and is mediated by the cytoplasmic tail of the transporter. The small GTP-binding protein, RhoA and its downstream effector, Rho kinase regulate NHE3, possibly by controlling the level of myosin phosphorylation, that in turn determines the organization of actin. The cytoskeleton may not only be involved in the maintenance of the basal rate of transport, but is also likely to sense physical alterations and transmit signals to modulate NHE3 activity, thus providing fast and effective control of the exchanger .

Introduction

The Na^+/H^+ exchanger (NHE) family is essential for cellular pH homeostasis as well as transepithelial ion and water transport (for review see [1-3]). To date six mammalian isoforms have been identified (NHE1 to 6). All are integral membrane proteins that catalyze the

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electroneutral exchange of Na^+ for H^+. They show considerable structural homology, but their differential tissue distribution suggests that each isoform performs a unique function.

NHE1, found in the plasma membrane of most animal cells, is often referred to as the "housekeeping isoform", as it plays a central role in maintaining cytosolic pH and cell volume. NHE6, also a ubiquitous protein, is expressed exclusively in the mitochondria, where it seemingly regulates ion and volume homeostasis. The other isoforms show a more restricted tissue distribution. NHE3 is the exchanger of the apical membrane of epithelial cells in the kidney tubules and in the gastrointestinal tract [4, 5]. It catalyzes Na^+ entry into these cells, thereby contributing to transepithelial Na^+ (re)absorption. NHE3 is known to be regulated by a large variety of hormones (see [1]) as well as by physical parameters, including the cell volume [6, 7, 8]. The fine regulation of the activity of this isoform provides the adjustments necessary for the maintenance of systemic salt and fluid balance.

Regulation of NHE3

Several signaling systems affect NHE3 [9, 10], but the exact molecular mechanisms underlying the regulation remain unresolved. In the past years two major mechanisms have been invoked to account for the acute control of NHE3. First, the number of cell surface transporters can change through alterations in the rate of endocytosis and/or exocytosis. Second, the activity of the individual exchangers can also be regulated. After briefly summarizing other known mechanisms of regulation, the remainder of this chapter will focus on the cytoskeletal control of the transporter.

Changes in the surface expression of NHE3

Studies of the subcellular localization of NHE3 revealed the existence of a substantial intracellular pool of transporters [11, 12]. The exchanger undergoes continuous, phosphatidylinositol 3' kinase-dependent cycling between the plasma membrane and endosomal vesicles [13]. Its endocytosis occurs primarily via a clathrin-dependent fusion mechanism [14]. The number of transporters at the surface can be rapidly altered either by mobilization of additional molecules from subcellular pools or by excess retrieval from the plasma membrane. Indeed, decreased surface expression of NHE3 was shown in association with inhibition of protein kinase C [PKC] [15] or protein kinase A [PKA] [16]. Conversely, increased abundance at the surface was associated with stimulation by basic fibroblast growth factor [17].

Although this is a powerful means of control, alterations in transport are not always accompanied by changes in expression at the cell surface suggesting that modulation of the transport at the level of individual transporters represents a distinct regulatory mechanism.

Regulation by phosphorylation

Acute regulation of NHE3 has been linked to phosphorylation [18-21]. Indeed, the primary structure of the protein indicates the presence of several potential phosphorylation sites for both PKC and PKA.

Hormones that elevate cAMP levels are potent inhibitors of NHE3. The exchanger is phosphorylated under basal conditions, and this phosphorylation is further increased after PKA activation [18-20]. Despite these results, there is controversy both about the role of phosphorylation in the inhibition of NHE3 and concerning the particular phosphorylation sites involved. Mutation of the primary target of PKA phosphorylation reduces the inhibitory effect only partially [19], suggesting that direct phosphorylation of NHE3 might not be the sole mechanism of regulation.

NHE3 is linked to accessory proteins

Search for accessory proteins involved in the regulation of NHE3 led to the identification of Na^+/H^+ exchanger regulatory factors (NHERF) [22]. Currently two homologous proteins that associate with the exchanger are known: NHERF1, also known as ezrin binding protein 50 (EBP50), and NHERF2, which is also called E3KARP/TKA1 [23, 24]. Immunoprecipitation experiments carried out in PS120 cells transfected with both NHE3 and NHERF showed physical and functional association of the two proteins. NHERFs contain two PDZ domains, which in other systems mediate specific protein-protein interactions. Their presence suggests that NHERFs might serve as adapters, nucleating protein complexes. Indeed, NHERF1 was shown to bind to ezrin, an actin-binding protein of the ERM (ezrin/radixin/moesin) family [26]. ERM family members link membrane proteins with the actin cytoskeleton, and ezrin is

also a protein kinase A-binding protein (AKAP) [27]. In PS120 cells interaction of NHE3, NHERF and ezrin was suggested to be essential for PKA-mediated phosphorylation and inhibition of NHE3 activity [24]. A model based on these data postulates that NHERFs act as scaffolding proteins and the signaling complex containing NHE3, NHERF and ezrin mediates regulation of Na^+/H^+ exchange.

Cytoskeletal regulation of NHE3

The actin-based cytoskeleton is a dynamic and highly organized structure composed of filamentous (F) actin and associated proteins, regulating the assembly, length and stability of actin filaments. One of the actin-associated proteins is myosin, which in non-muscle cells has a role in adhesion, in reinforcing the submembranous actin to maintain cell shape, and in promoting motility. Association with actin is controlled by the phosphorylation of myosin light chain, a key event in modulating cell contractility and the organization of the cytoskeleton.

Normal NHE3 function requires an intact actin cytoskeleton

In order to establish the role of the cytoskeleton in NHE3 function, we investigated the effect of actin modifier agents on the transporter. The study of NHE3 is complicated by the fact that most epithelial cells express more than one type of plasmalemmal NHE. To overcome this problem, we used a CHO cell line (AP1 cells) that was engineered to lack endogenous NHE [28]. NHE3 was stably expressed in this cell line and its activity was assessed by monitoring intracellular pH using a fluorescent pH-sensitive dye, BCECF. After acidification of the cytosol by the ammonium prepulse technique, reintroduction of Na^+ activates NHE3, leading to normalization of the pH. To test the effect of actin disruption, we used drugs that are known to interfere with the normal organization of the cytoskeleton [13]. Cytochalasin D is a fungal metabolite that disturbs the ongoing cycle of F-actin polymerization/depolymerization by binding to the fast-growing (barbed) end of the filament, thereby preventing the addition of further monomers [29]. This results in gradual disappearance of normal actin stress fibres and marked alteration of cell morphology. We found this drug to induce a profound inhibition of the function of NHE3. Interestingly, the effect proved to be isoform-specific, as NHE1 was not inhibited by cytochalasin treatment. These effects on NHE3 were replicated using a second antagonist of actin filament formation. Latrunculin A interferes with actin organization by binding and sequestering G-actin, thereby preventing the incorporation of monomers into F-actin filaments [30]. Like cytochalasin, this drug also reduced the activity of NHE3.

Evidence for a physical link between actin and NHE3

We also obtained data by both biochemical and immunofluorescence methods that suggest a physical link between the exchanger and actin [13]. The cytoskeletal proteins are known to be largely insoluble in mild nonionic detergents. In CHO cells approximately 40% of the total cellular actin remains insoluble after extraction with 0.5% Triton X-100. Under these conditions about 10% of the total cellular NHE3 is also insoluble, suggesting a link to the cytoskeleton. Disruption of the normal F-actin architecture reduced the amount of insoluble actin and also caused a decrease in the amount of precipitable (cytoskeleton-associated) NHE3.

Subcellular colocalization was studied by simultaneous immunostaining of the exchanger and actin. As discussed above, under basal conditions NHE3 is localized both to the plasma membrane and in recycling vesicles. The diffuse distribution throughout the cell makes colocalization with actin difficult to ascertain. However, following cytochalasin treatment F-actin collapses and forms large subcortical aggregates. Under these conditions NHE3 appears to accumulate in the regions where actin concentrates, suggesting a physical interaction [13].

Cytoskeletal regulation of NHE3 requires a domain in the cytoplasmic tail

The analysis of truncated mutants of NHE3 and chimeras constructed from NHE3 and NHE1 revealed certain structural requirements for cytoskeletal regulation [13]. A chimera made up of the N-terminal domain of NHE3 and the cytosolic (C-terminal) part of NHE1 behaves like NHE1, *i.e.* it is not sensitive to cytochalasin.

In contrast, the molecule possessing the N-terminal section of NHE1 and the cytoplasmic C-terminus of NHE3 is inhibited by cytochalasin D, unlike the wildtype NHE1. A series of truncation mutants was used to further localize the region that is important for cytoskeletal regulation. The domain encompassed by residues 650 and 684 proved to be essential for the cytoskeletal control of the transporter. Interestingly this region overlaps with that defined to mediate the interaction of NHE3 with NHERF2 [31]. It also contains a domain (RKRL, residues 656-659) similar to the sequence of other membrane proteins that bind ERM family members [32].

RhoA and the control of NHE3 activity

Small GTP-binding proteins of the Rho family are major regulators of the actin cytoskeleton [33]. In most cells, activation of RhoA itself is associated with stress fibre and focal adhesion formation, while the two other prominent members of the family, cdc42 and Rac, are involved in the organization of membrane filopodia and ruffles, respectively. In order to study the effects of these proteins on NHE3, we introduced inactive forms of Rac, cdc42 and RhoA by transfection and monitored Na^+/H^+ exchange activity in individual transfected cells [34]. Cotransfection with green fluorescent protein (GFP) was used to identify transfected cells, followed by loading with BCECF and monitoring internal pH changes by single cell imaging. We found no evidence for control of NHE3 activity by Rac or cdc42. In contrast, dominant-negative RhoA consistently decreased NHE3 activity. A similar inhibition was seen with the dominant-negative form of its downstream effector, Rho kinase, suggesting that phosphorylation reactions may be involved.

Myosin phosphorylation is a prerequisite for NHE3 function

Factors regulating myosin phosphorylation affect both cell morphology and the contractility of the cytoskeleton. Myosin light chain kinase is primarily, but not exclusively responsible for the phosphorylation of the light chain. Rho kinase promotes myosin phosphorylation by two distinct mechanisms: by direct phosphorylation of the light chain and also by inhibition of its dephosphorylation [35, 36]. When these two kinases are blocked by pharmacological inhibitors, myosin undergoes rapid dephosphorylation. Concomitantly, the activity of NHE3 drops markedly [34]. Therefore myosin phosphorylation seems to be a requisite for optimal function of NHE3, probably due to its major role in cytoskeleton organization. RhoA may affect NHE3 indirectly, by regulating myosin phosphorylation. Fig. 1 offers a schematic summary of the pathways that may link RhoA and NHE3.

What is the mechanism of cytoskeletal regulation of NHE3?

Cytoskeletal regulation does not involve changes in the number of surface exchangers.

The cytoskeleton is known to be involved in endocytosis and exocytosis. It is therefore possible that normal traffic of NHE3 in and out of the cells requires an intact actin skeleton. In this event, the observed inhibitory effects of actin disruption might result from the reduced expression of exchangers at the cell surface. To investigate this possibility, we studied the subcellular distribution of NHE3 following treatment of the cells with F-actin antagonists [34, 13]. Using cells expressing NHE3 with an exofacial tag we quantified surface NHE3 and showed that the number of transporters present on the surface was unchanged after disruption of the actin cytoskeleton.

With these experiments we ruled out the possibility that the actin cytoskeleton or myosin phosphorylation control NHE3 transport by modulating its intracellular trafficking. Thus the cytoskeleton must influence the intrinsic activity of the transporter itself. The molecular mechanisms of this regulation remain incompletely understood, but several possibilities can be considered. Physical linkage to the cytoskeleton might be essential to maintain the active conformation of the protein. On the other hand, NHE3 might be fully active only in special subdomains of the cell and its localization to these regions may depend on the cytoskeleton. In polarized epithelial cells the apical membrane, where NHE3 resides, might contain protein or lipid factors necessary for optimal activity of the exchanger. This discrete cellular localization might also explain the unique pattern of regulation of this isoform. Further investigation is needed to clarify the detailed mode of cytoskeletal regulation.

Is the cytoskeleton involved in the regulation by cAMP?

The role of the cytoskeleton might be more than just maintaining basal activity of NHE3. It could convey regulatory signals to the transporter, providing a fast and efficient coupling system to deliver signals from receptors and other sources. Here we consider the possible role of the cytoskeleton in the transduction of regulatory signals.

As discussed earlier, one of the most potent inhibitory stimuli for NHE3 is the elevation of cAMP. Despite the large amount of data documenting this regulation, the underlying molecular mechanism is still imperfectly understood. Direct phosphorylation of the exchanger by protein kinase A does not seem to provide a sufficient explanation, since a mutant NHE3 lacking serine 605, and exhibiting no detectable increase in phosphorylation upon PKA activation shows only partially reduced inhibition by cAMP [19]. What is the mechanism of the phosphorylation-independent inhibition?

Although we did not detect PKA-induced phosphorylation of this mutant, it is conceivable, that other sites are phosphorylated and can substitute for the

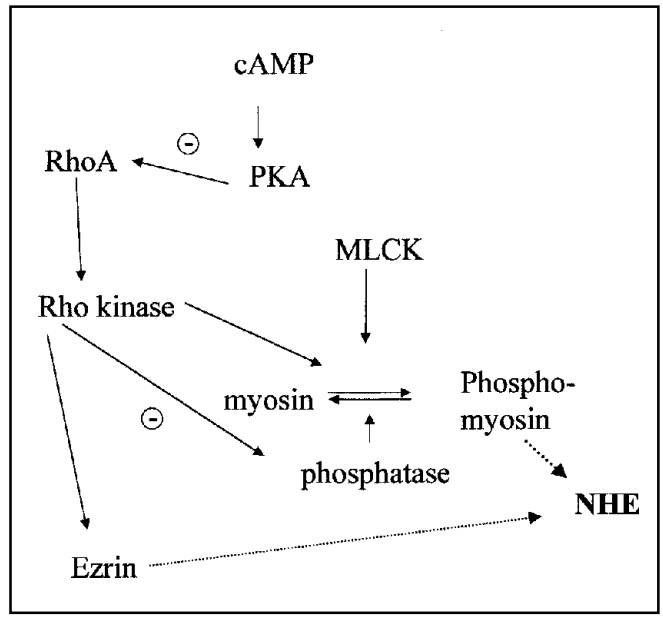

Fig 1. *Scheme summarizing the pathways that may link RhoA and NHE3.*

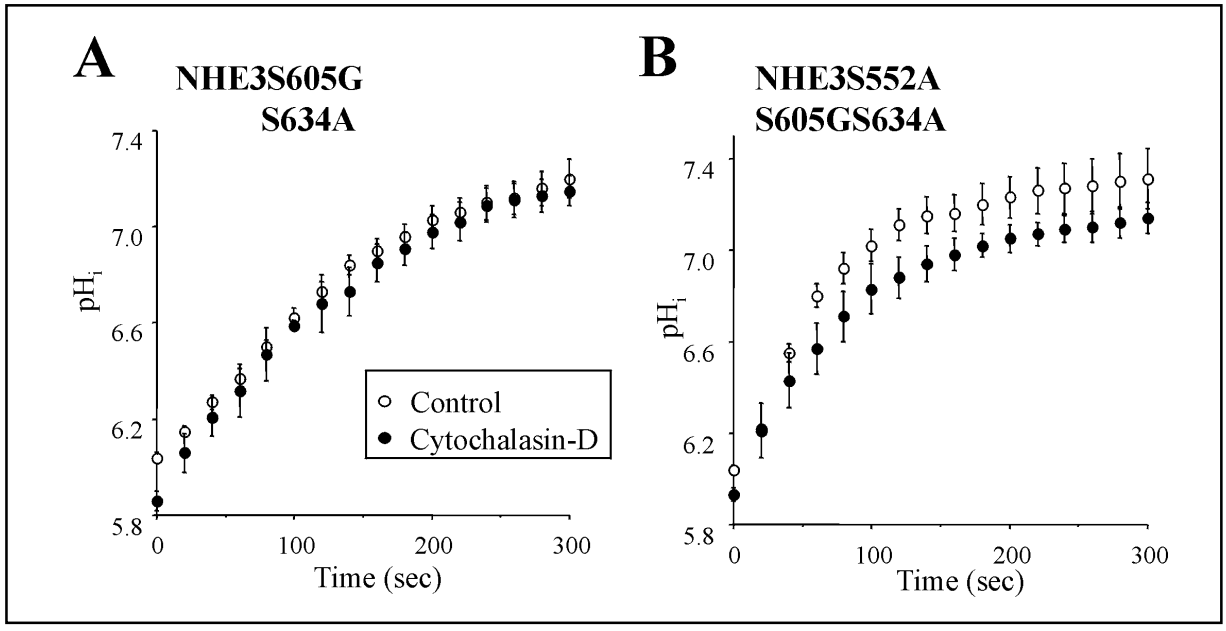

Fig 2. *Effect of actin disruption on the activity of mutant NHE3.* AP-1 CHO cells expressing mutant NHE3 lacking serine 605, 634 (A) or serine 605, 634 and 585 (B), were treated without (open circles) or with (solid circles) 10 µM cytochalasin D for 30 min. In the last 10 minutes of the treatment, they were loaded with BCECF-AM in bicarbonate-free isotonic medium in the presence of 50 mM NH_4Cl. The cells were then washed with a Na^+-free medium, and their pH_i was monitored fluorimetrically. Recording was initiated upon reintroduction of extracellular Na^+ to induce Na^+/H^+ exchange. The results are means ± S.E. of 6 determinations from 3 separate experiments.

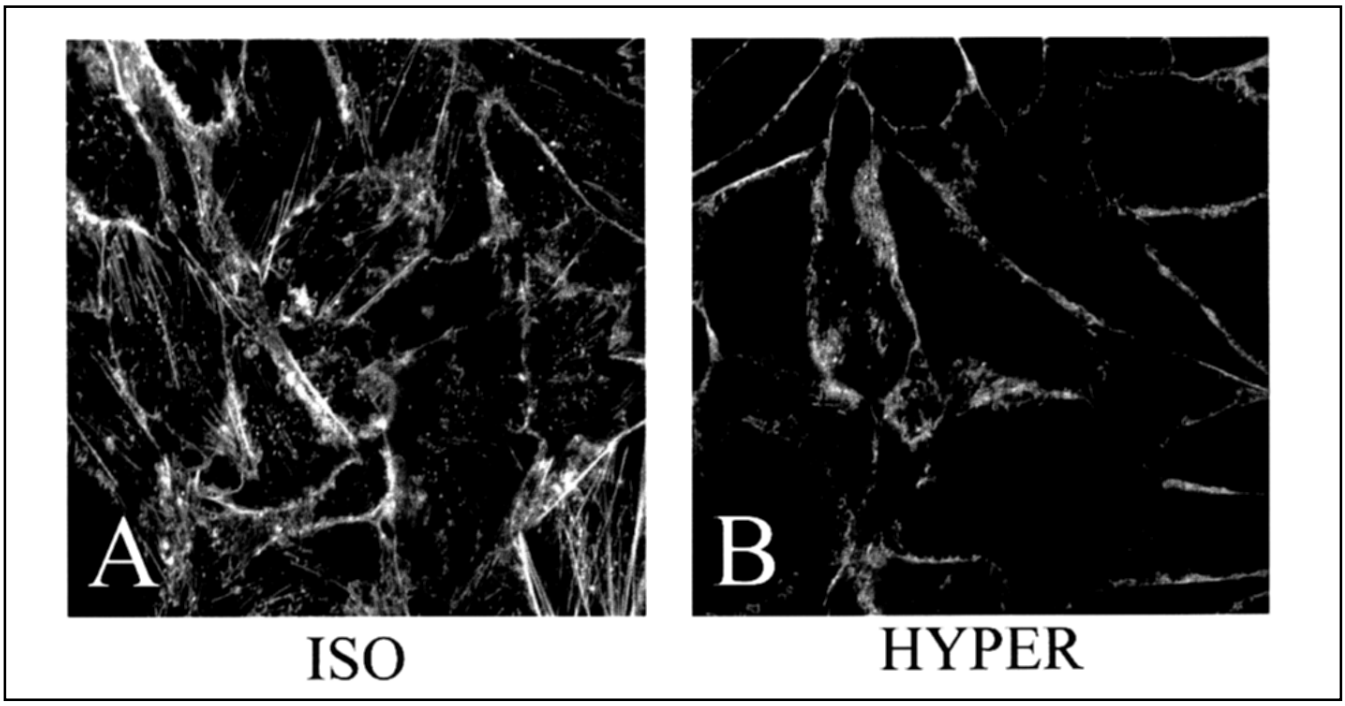

Fig 3. *Effect of cell shrinkage on the actin cytoskeleton.* CHO cells were incubated in isoosmotic (A, ISO) or hyperosmotic (isoosmotic supplemented with 200 mOsm sucrose, 500 mOsM total osmolarity) (B, HYPER) medium for 10 min at 37 ºC. The cells were then fixed, permeabilized and stained with rhodamine-phalloidin to visualize F-actin. Images were acquired using a confocal microscope.

mutant serine. Zhao et al [20] found another site, serine 552 to be essential for transport inhibition by PKA. The interpretation of these data is somewhat complicated by the fact that these serines are localized in a region that may be involved in the interaction of NHE3 with NHERF. In addition to abolishing a potential phosphorylation site, mutations in this region could disturb the structural integrity of the protein, altering its interactions with possible regulators. Phosphorylation of ancillary proteins might also occur and be a factor in conveying the inhibition.

Elevation of cAMP is known to cause marked changes in the cytoskeleton. Indeed, this effect is thought to be an important factor in the PKA-induced regulation of the $Na^+/K^+/Cl^-$ cotransporter [37], Na/P_i exchanger [38] and CFTR [39]. Notably, the effects of actin-modifier drugs and cAMP on the cytoskeleton and the transporter are remarkably similar. Both induce loss of stress fibres and cell rounding, and potently reduce NHE3 activity. Further, as shown in Fig. 2, mutant NHE3 molecules, lacking potential phosphorylation sites in two (605 and 634) or three (552, 605 and 634) places are no longer inhibited by either cytochalasin D or cAMP (not shown). This observation points to a likely common mechanism of inhibition. Thus, it is conceivable that modification of the cytoskeleton plays a role in mediating NHE3 inhibition. Our preliminary data indeed show that transfection of constitutively active RhoA partly prevents inhibition of the transporter by cAMP.

Is the cytoskeleton involved in regulation of NHE3 by cell volume?

Responsiveness to changes in cell volume seems to be a general property of Na^+/H^+ exchangers. Interestingly, the different isoforms of the exchanger behave in opposite ways [6]. NHE3 is markedly inhibited by cell shrinkage, while NHE1 is activated under the same conditions. The mechanism whereby cell volume affects the NHEs is still undefined. During the last years a number of potential signaling pathways were excluded. Although cell shrinkage leads to activation of several kinases, inhibitors of protein kinase C, Ca-calmodulin dependent protein

kinase [7] or tyrosine kinases [40] do not interfere with the osmotic inhibition of the exchanger.

Modulation of the cytoskeleton by cell volume changes might mediate inhibition of NHE3. The cytoskeleton has long been implicated in sensing and mediating mechanical stress. Indeed, a number of studies have suggested that the organization of the actin cytoskeleton is important for cell volume regulation (see [41]).

Little is known about the ways shrinkage regulates actin organization. Shrinkage induces an increase in F-actin content in Ehrlich ascites tumor cells [42] and in neutrophils [43]. Morover, in CHO cells tyrosine phosphorylation of cortactin, a protein of the cortical skeleton is increased upon shrinkage [44]. Visualization of actin indeed revealed a change in the organization of the cytoskeleton upon exposure to hyperosmolarity. Fig. 3 exemplifies the changes observed in CHO cells. After 10 minutes of incubation in a hyperosmotic medium, the cells show a decrease in stress fiber content, while the staining of cortical actin increases.

Recently cdc42, a member of the Rho family has been shown to translocate to the membrane fraction upon shrinkage [45]. This phenomenon is a sign of its activation and could represent the mechanism for the observed alterations in actin structure.

All these data imply that the cytoskeleton is indeed affected by changes in cell size and is therefore a good candidate for mediating inhibition of NHE3 activity under these conditions. Our future experiments are aimed at clarifying the role of the cytoskeleton in the osmotic regulation of NHE3.

In summary, NHE3 needs an intact actin cytoskeleton for its optimal activity. In addition to maintaining the basal transport rate of the exchanger, the actin skeleton could transmit regulatory signals modulating NHE3. Myosin might be a central player in this sequence, as its phosphorylation appears to alter the function of the exchanger. In this regard, several signaling pathways could affect NHE3 indirectly by controlling the state of myosin phosphorylation and/or the state of the actin cytoskeleton. This provides a fast and effective site of signal integration, where the activity of the exchanger would be adjusted according to the prevailing metabolic conditions.

Abbreviations

BCECF, 2',7'-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein; CHO: chinese hamster ovary; ERM, ezrin/radixin/moesin; GFP: green fluorescent protein; HA, hemagglutinin; MLCK, myosin light chain kinase; NHE, Na^+/H^+ exchanger; NHERF, NHE regulatory factor; pH_i, cytosolic pH; PKA; protein kinase A

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