Ca\(^{2+}\)-dependent Inhibition of Na\(^{+}/H^{+}\) Exchanger 3 (NHE3) Requires an NHE3-E3KARP-\(\alpha\)-Actinin-4 Complex for Oligomerization and Endocytosis

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Two PDZ domain-containing proteins, NHERF and E3KARP are necessary for cAMP-dependent inhibition of Na\(^{+}/H^{+}\) exchanger 3 (NHE3). In this study, we demonstrate a specific role of E3KARP, which is not duplicated by NHERF, in Ca\(^{2+}\)-dependent inhibition of NHE3 activity. NHE3 activity is inhibited by elevation of intracellular Ca\(^{2+}\) (Ca\(^{2+}\)) in PS120 fibroblasts stably expressing E3KARP but not those expressing NHERF. In addition, this Ca\(^{2+}\)-dependent inhibition requires Ca\(^{2+}\)-dependent association between \(\alpha\)-actinin-4 and E3KARP, NHE3 is indirectly connected to \(\alpha\)-actinin-4 in a protein complex through Ca\(^{2+}\)-dependent interaction between \(\alpha\)-actinin-4 and E3KARP, which occurs through the actin-binding domain plus spectrin repeat domain of \(\alpha\)-actinin-4. Elevation of [Ca\(^{2+}\)] results in oligomerization and endocytosis of NHE3 as well as in inhibition of NHE3 activity. Overexpression of \(\alpha\)-actinin-4 potentiates the inhibitory effect of ionomycin on NHE3 activity by accelerating the oligomerization and endocytosis of NHE3. In contrast, overexpression of the actin-binding domain plus spectrin repeat domain acts as a dominant-negative mutant and prevents the inhibitory effect of ionomycin on NHE3 activity as well as the oligomerization and internalization of NHE3. From these results, we propose that elevated Ca\(^{2+}\) inhibits NHE3 activity through oligomerization and endocytosis of NHE3, which occurs via formation of an NHE3-E3KARP-\(\alpha\)-actinin-4 complex.

Na\(^{+/H^{+}\) exchanger 3 (NHE3) mediates the majority of NaCl and NaHCO\(_3\) absorption in the ileum and proximal tubule of kidney (1–3). Elevation of [Ca\(^{2+}\)], induced by several physiologic and pathobiologic agonists (carbachol, serotonin, Escherichia coli heat-stable toxin b, rotavirus enterotoxin NSP5) inhibits NaCl absorption and brush border Na\(^{+}/H^{+}\) exchange activity in the small intestine and colon (4–7). However, the effect of elevation of [Ca\(^{2+}\)], in cell culture models differs among cell lines. In human colon cancer Caco-2 epithelial cells (C2bbe) stably transfected with NHE3, elevation of [Ca\(^{2+}\)], by treatment with thapsigargin inhibited NHE3 activity (8). In contrast, elevating [Ca\(^{2+}\)], by treatment with ionomycin did not alter NHE3 activity in PS120 fibroblasts (9), although basal [Ca\(^{2+}\)], is involved in the regulation of NHE3 activity in these cells in a calmodulin- or calmodulin kinase II-dependent manner (10). These results therefore suggest that a regulatory factor, which is specifically expressed in ileum and Caco-2 (C2bbe) epithelial cells but not in PS120 fibroblasts, might be required for the Ca\(^{2+}\)-dependent inhibition of NHE3 activity. To understand the mechanism of this inhibition, the molecular identity of the regulatory factors involved in the Ca\(^{2+}\)-dependent inhibition of NHE3 activity needed to be elucidated.

NHERF and E3KARP, two tandem PSD-95/Dlg-1/ZO-1 (PDZ) domain-containing proteins, were originally identified as regulatory proteins for protein kinase A (PKA)-dependent regulation of NHE3 (11–13). Both NHERF and E3KARP interact with NHE3 through their C-terminally extended second PDZ domain (P2C). In addition, the last 30 amino acids of these PDZ domain proteins interact with ezrin. Ezrin is thought to act as an A kinase-anchoring protein, which physically places PKA near NHE3 (14, 15). Either of these PDZ domain proteins is necessary for cAMP-induced inhibition of NHE3 activity by allowing PKA-dependent phosphorylation of NHE3 (16).

Another mechanism for acute regulation of NHE3 activity includes membrane trafficking between an intracellular recycling compartment and the plasma membrane (17–19). NHE3 is active on the plasma membrane, and the alteration of surface NHE3 protein abundance plays a role in regulation of NHE3 activity by extracellular agonists including peptide hormones, growth factors, and neurotransmitters (1). However, it is still unknown whether NHERF or E3KARP is involved in regulation of NHE3 trafficking. Whether either NHERF or E3KARP is involved in the Ca\(^{2+}\)-dependent inhibition of NHE3 activity is also unknown.

\(\alpha\)-Actinin is a class of actin-binding protein that cross-links F-actin bundles or networks and also connects F-actin to the plasma membrane. So far, four isoforms (\(\alpha\)-actinin-1–4) of human \(\alpha\)-actinin have been identified. These are classified into (i) muscle type (\(\alpha\)-actinin-2 and -3) and (ii) non-muscle type (\(\alpha\)-actinin-1 and -4). \(\alpha\)-Actinin exists as an antiparallel homodimer with a globular actin-binding domain, four spectrin-like repeats; aa, amino acids; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight.
like repeats, and two EF-hands motifs (20). Dimerization of α-actinin is mediated by its spectrin-like repeats (21). The C-terminal EF-hands domains differ among the α-actinin classes. Muscle isoforms contain non-functional EF-hand motifs, which do not bind Ca$^{2+}$ at physiological concentrations, and thereby bind to actin filaments in a Ca$^{2+}$-insensitive manner (20). In contrast, non-muscle isoforms contain two functional EF-hand motifs, and Ca$^{2+}$ binding to these EF-hand motifs reduces the affinity of α-actinin for F-actin (22). α-Actinin-4 (ACTN4) originally was identified as a protein that is up-regulated upon enhanced cell movement and is related to cancer invasion (23). ACTN4 requires a Ca$^{2+}$-dependent association between E3KARP and ACTN4. This is the first study to show that E3KARP plays a unique role in regulation of NHE3 activity, which is not duplicated by NHERF.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse monoclonal (9E10) antibody against Myc epitopes was from Babco Inc. (Berkeley, CA), and monoclonal anti-NSV-G antibody (body PSD4 (hydromeda culture medium) was kindly provided by Prof. D. Louvard (Curie Institute, Paris, France). The rabbit polyclonal anti-NHERF antibody, which has been reported to specifically recognize NHERF not α-actinin-1 (23), and the cDNA for ACTN4 were kindly provided by Dr. S. Hirohashi (National Cancer Center Research Institute, Tokyo, Japan). Dulbecco's modified Eagle's medium was from Invitrogen. Tetramethylammonium was from Fluka Chemical Corp. (Milwaukee, WI). Ionomycin and thapsigargin were from Sigma. Immobilized GST fusion proteins were from Ambion (Austin, TX). Radioactive precursors were from Amersham Biosciences (Milwaukee, WI). Pepsin and collagenase were from Sigma. Protease inhibitor cocktail was from Roche Molecular Biochemicals, Indianapolis, IN. Matrix-assisted laser desorption/ionization was performed with an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). The fidelity of the PCR products was confirmed by nucleotide sequencing. The oligomerization and endocytosis of NHE3 require a Ca$^{2+}$-insensitive mannan-binding lectin-Menten kinetics. The S.E. was calculated by computer to reflect variability of the parameters estimated.

**Protein Identification by Peptide Mass Fingerprinting Analysis**—Rabbit ileal villus cells were lysed in buffer A (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 5 mM MgCl$_2$, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin), unbroken cells were removed by centrifugation at 10,000 × g for 10 min followed by centrifugation at 100,000 × g for 40 min. The pellets were treated with buffer A containing 1% Triton X-100 to solubilize membrane-associated proteins and centrifuged at 100,000 × g for 40 min to remove insoluble pellets. Immobilized GST fusion proteins were reacted with aliquots (5 mg) of the ileal membrane extracts and washed three times with buffer A containing 0.1% Triton X-100 prior to SDS-PAGE and protein digestion. The compatibility of ion-exchange chromatography and protein digestion was demonstrated with GST-fused proteins. The efficiency of protein digestion was determined by SDS-PAGE and Western blot analysis. The masses of the tryptic peptides were measured with a Voyager DE time-of-flight mass spectrometer (Perseptive Biosystems, Inc., Framingham, MA) at Pohang University of Science and Technology. Matrix-assisted laser desorption/ionization was performed with an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). The fidelity of the PCR products was confirmed by nucleotide sequencing. The oligomerization and endocytosis of NHE3 require a Ca$^{2+}$-insensitive mannan-binding lectin-Menten kinetics. The S.E. was calculated by computer to reflect variability of the parameters estimated.

**Immunoprecipitation and Immunoblot Analysis**—Co-immunoprecipitation experiments were performed using lysates from PS120/NHE3/V/E3KARP cells treated with ionomycin or vehicle. In some experiments, PS120/NHE3/V/E3KARP cells stably transfected with ACTN4 or ABDR14 were used for immunoprecipitation. Cells were lysed in buffer A containing 1% Triton X-100, followed by centrifugation at 100,000 × g at 4 °C for 15 min. Aliquots (1 mg of protein) of lysates were reacted with either anti-E3KARP or anti-ACTN4 antibodies for 1 h at 4 °C. Immune complexes were separated by binding to protein A-Sepharose resin and were washed three times with buffer A containing 0.1% Triton X-100 prior to SDS-PAGE. The amounts of E3KARP, ACTN4, and E3KARP in immune complexes were detected by Western blot analysis. The masses of the tryptic peptides were measured with a Voyager DE time-of-flight mass spectrometer (Perseptive Biosystems, Inc., Framingham, MA) at Pohang University of Science and Technology. Matrix-assisted laser desorption/ionization was performed with an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). The fidelity of the PCR products was confirmed by nucleotide sequencing. The oligomerization and endocytosis of NHE3 require a Ca$^{2+}$-insensitive mannan-binding lectin-Menten kinetics. The S.E. was calculated by computer to reflect variability of the parameters estimated.

**Measurement of Surface NHE3 Antigen**—To measure surface NHE3, PS120 cells were treated with either agonist or vehicle at room temperature under the same condition used for measurement of NHE3 activity and then surface-labeled with biotin as described previously (27). After rinsing in phosphate-buffered saline (150 mM NaCl and 2 mM KCl, pH 7.4), cells were treated with NHS-SS-biotin (0.5 mg/ml) for 30 min in borate buffer (154 mM NaCl, 10 mM boric acid, 7.2 mM Na$_2$HPO$_4$, pH 7.4) and exposed to the quenching buffer (20 mM Tris·HCl and 120 mM NaCl, pH 7.4). Cells were lysed in 1 ml of PBS containing 0.1% Triton X-100 and then fixed for 10 min with 3% glutaraldehyde. Immunoprecipitation and immunoblot analysis were performed using Protein A/Sepharose resin or affinity-purified polyclonal antibody. The efficiency of cell surface biotinylation of NHE3 was estimated to be at least 85% (28).

**Immunocytochemistry**—PS120 fibroblasts were plated on glass coverslips 24 h prior to experiments. After 18 h, monolayers were washed with serum-free medium and cultured for 4 h in serum-free medium. Cells were treated with isocitrate or vehicles for the indicated times, washed twice with ice-cold PBS, and then fixed for 10 min with 3% parformaldehyde in phosphate-buffered saline (PBS) buffer. The fixed cells were washed with PBS buffer, permeabilized for 10 min with PBS buffer containing 0.2% Triton X-100, and then blocked in blocking solution (PBS containing 10% fetal bovine serum) for 1 h at room temperature. Primary antibodies were incubated for 1 h at room temperature in
blocking solution at the following dilutions: 1:500 for polyclonal antibody Ab2570 (anti-E3KARP antibody) and 1:200 for polyclonal anti-ACTN4 antibody. Cells were then washed three times with PBS and incubated with fluorochrome-conjugated secondary antibodies. Cells were washed three times with PBS and mounted with Prolong Antifade (Molecular Probes, Inc.) and then examined with a Zeiss LSM410 confocal fluorescence microscope.

RESULTS

E3KARP Is Required for Inhibition of NHE3 Activity by Elevation of \([\text{Ca}^{2+}]_i\) — NHE3 and its regulatory proteins, NHERF and E3KARP, localize in BB membrane of ileum (29). Elevation of \([\text{Ca}^{2+}]_i\) induced by treatment with carbachol or ionomycin equivalently inhibit BB \(\text{Na}^+ / \text{H}^+\) exchanger activity in intact ileum (4). We reported that either NHERF or E3KARP was necessary for cAMP-induced inhibition of NHE3 activity in PS120 fibroblasts. To determine whether NHERF or E3KARP was involved in the \([\text{Ca}^{2+}]_i\)-dependent inhibition of NHE3, we examined the effect of ionomycin in PS120/NHE3V/E3KARP cells (Fig. 1). Taken together, these results suggest that E3KARP but not NHERF is necessary for inhibition of NHE3 activity induced by elevation of \([\text{Ca}^{2+}]_i\).

Identification of ACTN4 as an E3KARP-specific Binding Protein from Rabbit Ileal Membranes — Both NHERF and E3KARP, localize in BB membrane of ileum (29). Elevation of \([\text{Ca}^{2+}]_i\), induced by treatment with carbachol or ionomycin equivalently inhibit BB \(\text{Na}^+ / \text{H}^+\) exchanger activity in intact ileum (4). We reported that either NHERF or E3KARP was necessary for cAMP-induced inhibition of NHE3 in PS120 fibroblasts. To determine whether NHERF or E3KARP was involved in the \([\text{Ca}^{2+}]_i\)-dependent regulation of NHE3, we examined the effect of ionomycin in PS120 cells stably expressing either NHERF or E3KARP. As shown in Fig. 1A, in PS120 cells stably expressing NHE3 (PS120/NHE3V), NHE3 activity was not affected by treatment with 2 \(\mu\text{M}\) ionomycin, consistent with a previous report (9). Ionomycin treatment of PS120/NHE3V cells overexpressing NHERF (PS120/NHE3V/NHERF) also did not alter NHE3 activity (Fig. 1B). In contrast, in E3KARP-overexpressing PS120 cells (PS120/NHE3V/E3KARP), ionomycin treatment (15 min at room temperature) caused a 35% decrease in \(V_{\text{max}}\) estimates (3932 \pm 219 \mu\text{M/s} for control versus 2557 \pm 621 \mu\text{M/s} for ionomycin-treated cells, \(p < 0.01\) (Fig. 1C). The inhibitory effect of ionomycin on NHE3 activity was completely prevented by co-treatment with EGTA to chelate extracellular free calcium. However, ionomycin also is a protonophore that could affect intracellular pH. To support that elevating \([\text{Ca}^{2+}]_i\), inhibits NHE3 activity, we determined the effect of thapsigargin, an endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor. Treatment with 10 nM thapsigargin also inhibits NHE3 activity to a similar extent as ionomycin in PS120/NHE3V/E3KARP cells (Fig. 1D). Taken together, these results suggest that E3KARP but not NHERF is necessary for inhibition of NHE3 activity induced by elevation of \([\text{Ca}^{2+}]_i\).

Identification of ACTN4 as an E3KARP-specific Binding Protein from Rabbit Ileal Membranes — Both NHERF and E3KARP are involved in the cAMP-dependent inhibition of NHE3 through ezrin-mediated formation of a protein complex that includes PKA II and NHE3 (12, 13, 15). In the current study, we demonstrated that E3KARP but not NHERF is specifically involved in the \([\text{Ca}^{2+}]_i\)-dependent inhibition of NHE3 (Fig. 1). These findings suggest a model in which a protein factor, which specifically interacts with E3KARP but not with NHERF, may be required for the \([\text{Ca}^{2+}]_i\)-dependent inhibition of NHE3 in PS120/NHE3V/E3KARP cells.

To identify E3KARP-binding proteins that do not interact with NHERF, we incubated membrane extract derived from rabbit ileal villus cells with immobilized GST-E3KARP, GST-
NHERF, and GST as described under “Experimental Procedures.” As shown in Fig. 2A, we found that a 100-kDa protein specifically binds to immobilized GST-E3KARP but not to GST nor GST-NHERF. In contrast, NHERF interacts with two distinct proteins with molecular masses of 140- and 95-kDa, supporting the specific interaction of E3KARP with the 100-kDa protein. The 100-kDa protein band was excised and "in-gel"-digested with trypsin. The resultant peptides were eluted and analyzed by matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) mass spectrometer (Fig. 2B). The masses, designated as P1–P11, were compared with proteins in the Swiss-Prot data base. As shown in Fig. 2C, 11 masses matched the calculated masses of tryptic peptides of ACTN4 (within 100 ppm) are indicated with an arrow. C, peptide sequences and observed monoisotopic masses of the tryptic peptides (P1–P11) from protein band 3, which matched the calculated tryptic peptide masses of ACTN4. D, localization of ACTN4 and E3KARP in the ileum. Immunofluorescence staining was carried out by either anti-ACTN4 or anti-E3KARP (Ab2570) polyclonal antibodies. E, immunoelectron microscopy demonstrated the localization of ACTN4 in the ileal BB membrane (arrowheads) and terminal web (arrows).

**ACTN4 and E3KARP Are Enriched in BB Membrane of Ileum**—In ileum, both NHE3 and E3KARP are primarily localized in the BB membrane of ileum (29). To clarify whether ACTN4 also localizes in the ileal BB membrane, we investigated the localization of ACTN4 in ileum by immunocytochemistry. As shown in Fig. 2D, ACTN4 is mostly localized in BB, even though it also exists in basolateral membrane. E3KARP shows a similar pattern of localization with ACTN4 in BB membrane (Fig. 2D). Immunogold staining using electron microscopy showed that most of ACTN4 localizes in the area of the terminal web, but there is also a significant portion of ACTN4 in BB (Fig. 2E). These results indicate that ACTN4 is in the same location of ileal Na⁺-absorbing cells as NHE3 and E3KARP.

**E3KARP Directly Interacts with ACTN4 via the C-terminally Extended Second PDZ Domain of E3KARP**—E3KARP contains two PDZ domains, and NHE3 binds to the P2C domain of E3KARP (15). To elucidate which domain of E3KARP is involved in the interaction with ACTN4, membrane extracts of ileal villus cells were reacted with each separate domain of E3KARP (PDZ1 and PDZ2, C terminus; and C-terminally extended second PDZ domain) fused to GST. The GST fusion proteins were constructed by PCR and purified as described under “Experimental Procedures.” Immunoblot analysis using anti-ACTN4 antibody shows that ACTN4 specifically binds to immobilized GST-E3KARP but binds neither GST-NHERF nor GST alone (Fig. 3A), consistent with the result shown in Fig. 2A. ACTN4 binds to the P2C fragment of E3KARP, which is the
**Regulation of NHE3 by E3KARP and α-Actinin-4**

**Fig. 4. Ca**\(^{2+}\)-dependent formation of a protein complex containing ACTN4, E3KARP, and NHE3 in vivo. PS120/NHE3V/E3KARP cells were treated with 2 μM ionomycin (+) or Me\(_2\)SO (−) for 10 min. Lysates were incubated with anti-ACTN4, anti-E3KARP, or preimmune serum, and the immunoprecipitates (I.P.) were immunoblotted with P5D4 (anti-NHE3), anti-ACTN4, and anti-E3KARP antibodies, respectively. Similar results were found in three identical experiments.

by using Ni\(^{2+}\)-NTA affinity column chromatography. As shown in Fig. 3B, in the absence of free Ca\(^{2+}\), E3KARP binds minimally to immobilized GST-ACTN4. In contrast, in the presence of Ca\(^{2+}\), E3KARP binds to immobilized GST-ACTN4, and the amount of bound E3KARP is increased in a Ca\(^{2+}\)-concentration-dependent manner. The binding of ACTN4 with E3KARP reaches near maximum at 1 μM [Ca\(^{2+}\)]. This suggests that the interaction between ACTN4 and E3KARP may be regulated by physiological elevation of [Ca\(^{2+}\)].

ACTN4 contains an actin-binding domain, four spectrin-like repeats, and two EF-hand domains (20). To clarify which domain of ACTN4 is involved in the interaction with E3KARP, we generated multiple fragments of ACTN4 as GST fusion proteins described in Fig. 3C. The fragments were expressed in *E. coli* and immobilized to GSH-agarose (Fig. 3D). The immobilized GST-fused fragments of ACTN4 were incubated with His\(_{6}\)-tagged E3KARP in the absence or presence of 1 μM [Ca\(^{2+}\)]. E3KARP binds to the actin-binding domain (ABD) of ACTN4, and the binding of E3KARP to immobilized GST-ABD is increased by addition of two spectrin-like repeats (ABDR12) (Fig. 3E). However, spectrin-repeat domains (R14) themselves do not interact with E3KARP, suggesting that the actin-binding domain is mainly involved in the interaction of ACTN4 with E3KARP. The GST fusion proteins containing EF-hand motifs, i.e. R14EF, R34EF, and EF, associate with E3KARP in the presence of Ca\(^{2+}\), but much lower amounts of E3KARP bind to the EF-hand domains containing fusion proteins compared with ABD and ABDR12. From these results, we suggest that the interaction between E3KARP and ACTN4 is primarily mediated by the ABD domain of ACTN4, and this interaction is Ca\(^{2+}\)-dependent.

**Calcium-dependent Interaction of NHE3, E3KARP, and ACTN4 from PS120 Fibroblasts**—In this study, we showed that E3KARP interacts with ACTN4 in a Ca\(^{2+}\)-dependent manner in *vivo* (Fig. 3B). This result raised the question whether E3KARP and ACTN4 associate in a Ca\(^{2+}\)-dependent manner in *vivo*. To evaluate this possibility, we performed in *vivo* co-precipitation studies of E3KARP and ACTN4 with elevated [Ca\(^{2+}\)]. As shown in Fig. 4, ACTN4 was co-immunoprecipitated with E3KARP from PS120/NHE3V/E3KARP cells, and E3KARP was also co-precipitated by anti-ACTN4 antibody. In *vivo*, the association between E3KARP and ACTN4 requires elevation of [Ca\(^{2+}\)], induced by ionomycin treatment, consistent with the finding that E3KARP interacts with ACTN4 in a Ca\(^{2+}\)-dependent manner in *vivo*. NHE3 was precipitated by anti-E3KARP antibody, and the amounts of NHE3 associated with E3KARP were not affected by elevating [Ca\(^{2+}\)]. Precipitation of ACTN4 specifically pull-downs NHE3 after the treatment of ionomycin, whereas NHE3 is not co-precipitated with

**Fig. 3. Ca**\(^{2+}\)-dependent interaction between E3KARP and ACTN4 and mapping of interaction sites. A, 5 mg of extract prepared from ileal membrane by solubilization with 1% Triton X-100 was incubated with 3 μg of immobilized GST fusion proteins including NHERF, E3KARP, and the fragments of E3KARP (P1, PDZ1; P2, PDZ2; C, C terminus; P2C, C-terminally extended second PDZ domain) as indicated. The amounts of ACTN4 bound to the GST fusion proteins were determined by Western blotting with anti-ACTN4 antibody. The results shown are those of a single experiment representative of three experiments performed with independent preparations. B, effect of calcium on the interaction of E3KARP with ACTN4. An aliquot (0.1 μg) of purified His\(_{6}\)-E3KARP was incubated with 3 μg of immobilized GST-ACTN4 protein in the presence of various free Ca\(^{2+}\) concentrations as indicated, set with a Ca\(^{2+}\)/EGTA buffer. The amounts of His\(_{6}\)-E3KARP bound to GST-ACTN4 were determined by Western blotting with anti-E3KARP antibody. Representatives of four independent experiments are shown. C, representative primary structure of GST-fused ACTN4 fragments showing various domains (ABD, actin-binding domain; R, spectrin repeat domain; EF, EF-hand motif) of ACTN4. D, the GST-fused ACTN4 fragments were purified using GSH-Sepharose, subjected to 12% SDS-PAGE, and visualized by Coomassie Brilliant Blue staining. E, mapping of domains of ACTN4 involved in the Ca\(^{2+}\)-dependent interaction with E3KARP. An aliquot (0.1 μg) of His\(_{6}\)-tagged E3KARP was reacted with 3 μg of the GST-fused ACTN4 fragments in the absence or presence of 1 μM free Ca\(^{2+}\) concentration. The amount of E3KARP bound to the immobilized GST fusion proteins was measured by Western blotting with anti-E3KARP antibody. Representatives of four independent experiments are shown.

same region required for the interaction with NHE3, whereas it does not interact with PDZ1, PDZ2, and C-terminal regions of E3KARP, indicating the specificity of the association between ACTN4 and the P2C region of E3KARP.

**ACTN4 Interacts with E3KARP in a Ca**\(^{2+}\)-dependent Manner—E3KARP but not NHERF is involved in the Ca\(^{2+}\)-dependent inhibition of NHE3 activity (Fig. 1), and E3KARP specifically binds with ACTN4 (Figs. 2A and 3A). ACTN4 contains two EF-hand domains that are involved in Ca\(^{2+}\) binding (20, 22). Therefore, we examined the effect of Ca\(^{2+}\) on the direct interaction between GST-ACTN4 and E3KARP. His\(_{6}\)-tagged E3KARP was expressed in *E. coli* and homogeneously purified

[Diagram showing the interaction between E3KARP and ACTN4 and mapping of interaction sites.](image-url)
ACTN4 in the absence of ionomycin (Fig. 4). These in vivo data suggest that NHE3 constitutively interacts with E3KARP in a Ca\(^{2+}\)-independent manner, and the protein complex containing NHE3 and E3KARP is likely to be linked to ACTN4 through the Ca\(^{2+}\)-dependent interaction between E3KARP and ACTN4. 

ACTN4 Is Necessary for the Calcium-dependent Inhibition of NHE3—ACTN4 interacts with E3KARP in a Ca\(^{2+}\)-dependent manner in PS120 cells. To explore whether ACTN4 is involved in the Ca\(^{2+}\)-dependent inhibition of NHE3, we stably expressed either whole ACTN4 or a fragment which covers the actin-binding domain plus the spectrin-like repeats (ABDR14) of ACTN4 in PS120/NHE3V/E3KARP cells. The amounts of ACTN4, ABDR14, or E3KARP expressed in PS120/NHE3V/E3KARP (ACTN4), and PS120/NHE3V/E3KARP/ABDR14 (ABDR14) cells were determined by Western blotting (I.B.) with anti-ACTN4 polyclonal antibody, anti-Myc (9E10), or anti-E3KARP (Ab2570) antibodies as indicated. The indicated cells were treated with 2 \(\mu\)M ionomycin for 10 min, and lysates were incubated with anti-VSV-G (P5D4) antibody for immunoprecipitation (I.P.) of NHE3. The amounts of NHE3, ACTN4, and E3KARP in the immune complexes were measured by Western blotting with anti-VSV-G, anti-ACTN4, and anti-E3KARP antibodies as indicated. Representatives of three experiments performed with independent preparations are shown. Effect of elevating [Ca\(^{2+}\)], was induced by ionomycin treatment on NHE3 activity in PS120/NHE3V/E3KARP (B), PS120/NHE3V/E3KARP/ACTN4 (C), and PS120/NHE3V/E3KARP/ABDR14 (D) cells. PS120 cells were treated with 2 \(\mu\)M ionomycin or vehicle, and NHE3 activity was measured as described in Fig. 1. These data were obtained from five similar experiments.

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Next, we determined the amounts of ACTN4 co-precipitated with NHE3 from these three cell lines, all studied after elevation of [Ca\(^{2+}\)], as shown in Fig. 5B. The amount of ACTN4 co-precipitated with NHE3 increased in PS120/NHE3V/E3KARP/ACTN4 cells compared with PS120/NHE3V/E3KARP cells. This suggests that exogenously expressed ACTN4 as well as endogenous ACTN4 is co-precipitated with NHE3. In addition, both ABDR14 and endogenous ACTN4 were co-precipitated with NHE3 from PS120/NHE3V/E3KARP/ABDR14 cells, although the amount of endogenous ACTN4 associated with NHE3 decreased compared with that from PS120/NHE3V/E3KARP cells. These results show that exogenously expressed ACTN4 and ABDR14 are physically linked to NHE3, and over-expression of ABDR14 can block the association of endogenous ACTN4 with NHE3.

Next, we examined the effect of elevation of [Ca\(^{2+}\)] on NHE3 activity in these three cell lines. All measurements were done under identical experiment conditions on the same day. Fig. 5 shows a typical experiment. In PS120/NHE3V/E3KARP cells, treatment with 2 \(\mu\)M ionomycin caused a 30% decrease in \(V_{\text{max}}\) (\(V_{\text{max}}\) control, 1880 ± 67 \(\mu\)M/s versus 1326 ± 75 \(\mu\)M/s for ionomycin-treated cells) (Fig. 5C). In PS120/NHE3V/E3KARP/ACTN4 cells, treatment with 2 \(\mu\)M ionomycin caused a larger 45% decrease in \(V_{\text{max}}\) of NHE3 activity from 1956 ± 61 \(\mu\)M/s for control cells to 1083 ± 61 in ionomycin-treated cells (Fig. 5D). This suggests that overexpression of ACTN4 potentiates the inhibitory effect of ionomycin on NHE3 activity. In contrast, in PS120/NHE3V/E3KARP/ABDR14 cells, ionomycin treatment results in only a 7% decrease in \(V_{\text{max}}\) (3116 ± 104 \(\mu\)M/s for control cells versus 2918 ± 137 \(\mu\)M/s for ionomycin-treated cells).
cells) (Fig. 5E). Thus, overexpression of ABDR14, which decreases the Ca\(^{2+}\)-dependent interaction of ACTN4 with E3KARP, blocks the inhibitory effect of ionomycin on \(V_{\text{max}}\) of NHE3. From these results, we suggest that ACTN4 is necessary for the Ca\(^{2+}\)-dependent inhibition of NHE3 activity.

**Elevated [Ca\(^{2+}\)]\(_i\) Causes Oligomerization and Internalization of NHE3 in PS120/NHE3V/E3KARP Cells**—NHE3 localizes in both the plasma membrane and intracellular compartments of PS120 cells (30). NHE3 recycles between the plasma membrane and intracellular compartments with a half-life of ~15 min (31), and stimulated endocytosis is involved in acute regulation of NHE3 activity (18). To examine whether the ionomycin-induced inhibition of NHE3 activity in E3KARP-overexpressing PS120 cells is caused by internalization of NHE3 from the plasma membrane, we measured the surface amount of NHE3 by biotinylation with Sulfo-NHS-SS-biotin. In PS120/NHE3V cells, NHE3 exists in a monomeric form with a molecular mass of 85 kDa and also as a dimeric form of ~170 kDa. There is also a small amount of oligomeric NHE3 in cell lysates (Fig. 6A). Densitometry values for each form of NHE3 in cell lysates were determined in the absence and in the presence of ionomycin. As shown in Fig. 6B, under basal conditions NHE3 exists 56 ± 6% in monomeric, 30 ± 6% in dimeric, and 14 ± 1% in oligomeric forms in PS120/NHE3V cells. Treatment with ionomycin did not affect the percentages of each form of NHE3 (Fig. 6B). The amount of NHE3 on the plasma membrane in the absence and in the presence of ionomycin was determined by quantitation of the densities of NHE3 protein bands from data in Fig. 6A, as described under "Experimental Procedures." In the absence of ionomycin, about 13% of total NHE3 localizes on the plasma membrane, and the percent of NHE3 on the plasma membrane was not affected by elevation of [Ca\(^{2+}\)]\(_i\) in PS120/NHE3V cells (Fig. 6C).

In contrast, in PS120/NHE3V/E3KARP cells, the distribution of NHE3 changed 15 min after [Ca\(^{2+}\)]\(_i\) elevation (Fig. 6A). The percent of monomeric form of NHE3 in cell lysates de-
creased after ionomycin treatment, whereas the percent of both dimeric and oligomeric forms of NHE3 increased (Fig. 6B). The densitometric measurements show that the percent of mono-
meric NHE3 decreases from 55 ± 3% for control to 32 ± 4% for ionomycin-treated cells (Fig. 6B), whereas the percent of the dimeric form increases from 28 ± 5 to 44 ± 8% and that of the oligomeric form from 17 ± 3 to 24 ± 4% after ionomycin treatment. These results show that E3KARP may be involved in the oligomerization of NHE3 induced by elevation of [Ca\(^{2+}\)].

In addition, NHE3 is internalized from the plasma membrane after ionomycin treatment in PS120/NHE3V/E3KARP cells. 12.7 ± 0.6% of total NHE3 localizes in the plasma membrane in the absence of ionomycin, which is very similar to that in PS120/NHE3V cells (Fig. 6C). However, the amount of surface NHE3 diminished to 6.9 ± 0.7% in PS120/NHE3V/E3KARP cells after ionomycin treatment (Fig. 6C). Taken together, the results indicate that E3KARP is involved in the internalization as well as in the oligomerization of NHE3 induced by the elevated [Ca\(^{2+}\)], and NHE3 activity may be regulated by both internalization and oligomerization of NHE3.

**Oligomerization and Internalization of NHE3 Is Dependent on ACTN4**—The inhibitory effect of ionomycin on NHE3 activity was potentiated by overexpression of ACTN4 and blocked by stable overexpression of the ABD14 fragment of ACTN4. To determine whether ACTN4 regulates NHE3 activity through affecting the oligomerization and internalization of NHE3, we measured the surface percent of NHE3 in PS120/NHE3V/E3KARP cells expressing either ACTN4 or ABD14. In PS120/NHE3V/E3KARP/ACTN4 cells, the percent of monomeric NHE3 decreases from 52 ± 3% for control to 18 ± 3% for ionomycin-treated cells, whereas the percent of the dimeric form increases from 32 ± 1 to 49 ± 6% and that of the oligomeric form from 16 ± 2 to 33 ± 3% after ionomycin treatment (Fig. 6B). The amount of surface NHE3 diminished from 13.8 ± 0.5 to 4.2 ± 0.3% in PS120/NHE3V/E3KARP/ACTN4 cells after ionomycin treatment (Fig. 6C). Thus, overexpression of ACTN4 increased the oligomerization and the internalization of NHE3 induced by elevation of [Ca\(^{2+}\)]. In contrast, the ionomycin-induced oligomerization of NHE3 could not be detected in cell lysates of PS120/NHE3V/E3KARP/ABD14 cells (Fig. 6, A and B). Moreover, the internalization of surface NHE3 was not induced in the ABD14-expressing cells (Fig. 6C), consistent with the result showing that ionomycin treatment has no effect on NHE3 activity in PS120/NHE3V/E3KARP/ABD14 cells. These results further suggest that ACTN4 is involved in both oligomerization and internalization of NHE3 induced by elevating [Ca\(^{2+}\)].

**Co-localization of ACTN4, E3KARP, and NHE3 on the Plasma Membranes and in the Intracellular Compartment after Elevation of [Ca\(^{2+}\)].**—The distribution of NHE3, E3KARP, and ACTN4 exogenously expressed in PS120 fibroblasts was determined using confocal microscopy. Cells were treated with 2 μM ionomycin for 10 min prior to fixation. In the absence of iono-
mycin, ACTN4 localizes along actin stress fibers and plasma membrane of PS120 fibroblasts (Fig. 7a1), consistent with previous reports of localization of ACTN4 (23). E3KARP is diffusely distributed throughout the cytosol with some diffuse staining along the plasma membrane as reported previously (15) (Fig. 7a2). Double staining showed that ACTN4 and E3KARP co-localize at the plasma membrane as well as some areas within the cytosol (Fig. 7a3). The distributions of ACTN4 and E3KARP were drastically affected after the treatment with 2 μM ionomycin. Both ACTN4 (Fig. 7b1) and E3KARP (Fig. 7b2) co-localized in large aggregates or clusters along the plasma membrane and in intracellular areas after ionomycin treatment (Fig. 7b3), with these effects were observed in at least 50% of cells.

The effect of ionomycin treatment on the distribution of NHE3 and E3KARP was also determined. In the absence of ionomycin, NHE3 exists along the plasma membrane as well as in a juxtanuclear location as reported previously (Fig. 7c1). After ionomycin treatment, NHE3 is distributed in surface and intracellular clusters (Fig. 7d1). Double staining of NHE3 and E3KARP showed that the distribution of NHE3 overlapped with E3KARP (Fig. 7d3) in clusters randomly distributed along the plasma membrane and throughout the cells. These results show co-localization of NHE3, E3KARP, and ACTN4 in the clusters formed after elevation of [Ca\(^{2+}\)].

**Overexpression of ABD14 Blocks the Formation of Clusters Containing NHE3, E3KARP, and ACTN4**—As shown in Fig. 5 and 6, overexpression of ABD14 blocked the inhibitory effect of elevating [Ca\(^{2+}\)], on NHE3 activity by interfering with the oligomerization as well as internalization of NHE3. Next, it was examined whether the distributions of NHE3, E3KARP, and ACTN4 were affected by stable expression of ABD14. The localization of ABD14 was determined by staining using anti-
Myc antibody. As shown in Fig. 7e1, in the absence of ionomy-
cin, the distribution of ABD14 in PS120/NHE3V/E3KARP/ ABD14 cells showed a similar distribution to ACTN4 as shown in PS120/NHE3V/E3KARP/ACTN4 cells. E3KARP ex-
ists along the plasma membrane and diffusely localizes in cytosol (Fig. 7e2), and overlaying the images of ABD14 and E3KARP shows that these two proteins are co-localized along the plasma membrane and in cytosol in the absence of ionomy-
cin as shown in Fig. 7e3. The distribution of ABD14 is moder-
ately affected by treatment with ionomycin. ABD14 primarily localizes along the plasma membrane, but it seems to also still exist in focal adhesion plaques rather than in clusters (Fig. 7f1). E3KARP exists in the plasma membrane and throughout the cytosol, but it does not form clusters on the plasma mem-
brane or the intracellular compartments with Ca\(^{2+}\) elevation (Fig. 7f2). Therefore, ABD14 and E3KARP co-localize along the plasma membrane but not in the intracellular compartments (Fig. 7f3). The distributions of NHE3 and E3KARP were studied in the same experimental conditions. NHE3 primarily exists in a juxtanuclear location but is also in the plasma membrane in the absence of ionomycin (Fig. 7g1). However, now the distribution pattern of NHE3 was not affected by ionomycin treatment (Fig. 7h1). After ionomycin treatment, E3KARP is predominantly present throughout the cytosol, and it is not localized in any clustered compartment (Fig. 7h2). Although NHE3 and E3KARP exist along the plasma mem-
brane, the staining of NHE3 and E3KARP does not show any clustered pattern after treatment of ionomycin. From these results, we suggest that overexpression of ABD14 interferes with the clustered distribution of NHE3 and E3KARP and therefore blocks the oligomerization and internalization of NHE3.

**DISCUSSION**

Inhibition of Na\(^{+}/H^+\) exchange induced by elevation of [Ca\(^{2+}\)] is involved in normal intestinal physiology and in the pathophysiology of diarrheal disease (5, 32). However, the molecular mechanism by which the BB Na\(^{+}/H^+\) exchanger activity is inhibited by elevation of [Ca\(^{2+}\)] has not been identified until now. Our results show that at least two additional mole-
cules, E3KARP and ACTN4, are necessary for inhibition of NHE3 activity induced by elevation of [Ca\(^{2+}\)]. The following lines of evidence support this conclusion. 1) Acute Ca\(^{2+}\) inhibi-
tion of NHE3 activity requires E3KARP but not NHERF. 2) E3KARP interacts with ACTN4 in a Ca\(^{2+}\)-dependent manner and thus physically links NHE3 to ACTN4. 3) A large plasma
membrane protein complex forms after [Ca\(^{2+}\)]
release which contains E3KARP, ACTN4, and NHE3. This complex is required for the oligomerization of NHE3. 4) Elevation of [Ca\(^{2+}\)]
induces the redistribution of NHE3, E3KARP, and ACTN4 from plasma membrane to clusters localized on plasma membrane and in intracellular compartments. 5) Overexpression of either ACTN4 or ABDR14 affects the Ca\(^{2+}\)-dependent inhibition of NHE3 activity by modulating the Ca\(^{2+}\)-induced oligomerization and internalization of NHE3. ABDR14 is a dominant-negative ACTN4 mutant that prevents elevated [Ca\(^{2+}\)]
-induced oligomerization and internalization of NHE3.

NHERF and E3KARP, which have two PDZ domains and an ERM-binding domain, are closely related proteins sharing 52% amino acid identity, and both of them appear equivalently involved in cAMP-dependent inhibition of NHE3 activity in PS120 fibroblasts (12, 13). Although it initially appeared that NHERF and E3KARP might have redundant functions, specific function of E3KARP has recently suggested from the studies (33–35) that E3KARP but not NHERF specifically interacts with a plasma membrane Ca\(^{2+}\) ATPase isoform 2b, phospholipase C-β3, and serum and glucocorticoid-regulated kinase 1. Through interacting with these proteins, E3KARP may be involved in the Ca\(^{2+}\) influx mediated by Ca\(^{2+}\) ATPase 2b and phospholipase C-β3 (33, 35). In addition, the specific interaction of E3KARP with serum and glucocorticoid-regulated kinase 1 is required for the glucocorticoid-induced activation of NHE3 activity (34). In this current study, another specific role for E3KARP, which is not duplicated by NHERF in the same cell model, was demonstrated in the inhibition of NHE3 induced by elevated [Ca\(^{2+}\)]. The inhibitory effect of elevating Ca\(^{2+}\) on NHE3 activity was originally reported in Na\(^{+}\)-absorptive intestinal cells, which contain both NHERF and E3KARP in brush border. In small intestine, Na\(^{+}/H\(^{+}\) exchanger activity was reduced by elevation of [Ca\(^{2+}\)], by treatment with carbachol and ionomycin (4). In addition, elevation of [Ca\(^{2+}\)], by treatment with thapsigargin resulted in inhibition of NHE3 activity in C2bbe cells, a subclone of Caco-2 intestinal epithelial cells (8), which also have both NHERF and E3KARP (34). However, the inhibition of NHE3 activity by elevating Ca\(^{2+}\) was not reproduced in PS120 fibroblasts (9), which lack E3KARP, but was reconstituted in these cells by stable expression of E3KARP (Fig. 1). Taken together, these results suggest that E3KARP is required for the inhibition of NHE3 activity by elevating Ca\(^{2+}\) and that NHERF and E3KARP can serve distinct functions even when they occur in the same cells, for instance, in Na\(^{+}\) absorptive cells of the small intestine. It will be of interest to test whether elevated [Ca\(^{2+}\)] regulates NHE3 activity via formation of the protein complexes containing α-actinin-4/E3KARP in the small intestine.

The specificity of E3KARP in Ca\(^{2+}\)-dependent inhibition of NHE3 results from the specificity of the Ca\(^{2+}\)-dependent interaction of E3KARP with ACTN4 (Fig. 3). The extended second PDZ domain of E3KARP is primarily involved in the specific association with the actin-binding domain of ACTN4. Consistent with our findings, recent reports (24, 36, 37) have suggested the association of α-actinin with several PDZ domain-containing proteins. Non-muscle type α-actinin-1 and -4 associate with the PDZ domain of CLP-36 (24). In addition, α-actinin-2 interacts through its spectrin-like repeats and C-terminal region with the PDZ domain of ALP and ZASP/Cypher1 (36, 37),

**Fig. 7.** Effect of elevating [Ca\(^{2+}\)] on cellular distribution of NHE3, E3KARP, and ACT4 in PS120 fibroblasts expressing ACTN4 or ABDR14. A, PS120/NHE3V/E3KARP/ACTN4 cells were serum-starved for 4 h, and immunofluorescence staining was carried out under basal conditions (−) or after incubation with 2 μM ionomycin for 10 min (+). ACTN4, NHE, and E3KARP were double-stained with anti-Myc (9E10) (a1 and b1), anti-VSV-G (c1 and d1), and anti-E3KARP (a2, b2, c2, and d2) antibodies and analyzed by confocal microscopy using a ×40 lens. The overlaid images of the double staining are shown (e3, f3, c3, and d3). B, in PS120/NHE3V/E3KARP/ABDR14 cells, the distributions of ABDR14, NHE3, and E3KARP were determined by using anti-Myc (e1 and f1), anti-VSV-G (g1 and h1), and anti-E3KARP (e2, f2, g2, and h2) antibodies, respectively. The overlaid images of the double staining are shown (e3, f3, g3, and h3). Representatives of four independent experiments are shown.
Regulation of NHE3 by E3KARP and α-Actinin-4

respectively. Although these PDZ domain proteins interact with different regions of α-actinin, taken together, these results suggest a widely used coupling of isoforms of actinin with PDZ domain proteins.

In basal conditions, NHE3 primarily exists in monomeric and dimeric forms as revealed by separation on SDS-PAGE gels. Elevating Ca\(^{2+}\) decreased the total amount of cellular monomeric NHE3 while increasing the amounts of total dimeric and oligomeric NHE3. In contrast, transferrin receptor, which recycles between recycling endosome and plasma membranes as NHE3, does not oligomerize after elevation of [Ca\(^{2+}\)], (data not shown). These results provide the first evidence that elevating [Ca\(^{2+}\)], or any signal transduction for that manner, specifically shifts NHE3 into oligomers. Homodimerization of NHE3 has been shown to be mediated by the transmembrane regions of NHE3 in basal condition (9). In addition to NHE3, homodimerization of both E3KARP and ACTN4 have been suggested, and the PDZ domains of E3KARP and spectrin homology repeats of ACTN4 have been implicated in their homodimerization, respectively (21, 38). The co-immunoprecipitation of NHE3, E3KARP, and ACTN4 from PS120 fibroblasts after elevation of [Ca\(^{2+}\)], supports the idea that these three proteins are included in a protein complex. Taken together with our findings, one model of the NHE3 oligomerization could involve dimeric E3KARP bringing two NHE3 molecules to the complex with the anti-parallel homodimer ACTN4 binding to two E3KARP molecules (and consequently 4 NHE3 molecules), although the stoichiometry for the oligomerization has not been clarified. In this study, we found that both NHE3 and ACTN4 bound to the same region (P2C) of E3KARP. One possible explanation is that NHE3 and ACTN4 bind to different sites of the P2C fragment or to different E3KARP molecules in the same NHE3-containing complex. Therefore, to verify the stoichiometry of the protein complex, it will be necessary to elucidate the binding sites for either NHE3 or ACTN4 in the P2C fragment. Whereas only 10–15% of NHE3 is on the plasma membrane under basal conditions, the percent of total NHE3 that undergoes multimerization is larger. Given that the half-life of surface NHE3 is ~15 min (31) suggests that the likely explanation is that the newly trafficking NHE3 undergoes the same changes as that initially present on the plasma membrane.

The involvement of ACTN4 in formation of the NHE3 oligomers is supported by the findings that overexpression of ACTN4 increases the Ca\(^{2+}\)-dependent oligomerization of NHE3. In contrast, overexpression of ABDR14, which is the major part of ACTN4 involved in the Ca\(^{2+}\)-dependent interaction with E3KARP, inhibits oligomerization of NHE3 by competing with endogenous ACTN4 for E3KARP association. This dominant-negative construct lacks the EF-hands domain and suggests that EF-hands domains are necessary for the oligomerization/endocytosis of NHE3. Given the Ca\(^{2+}\)-binding function of EF-hands domains (20) and that elevated Ca\(^{2+}\) is necessary for the formation of the NHE3 complex, we speculate that a local elevation in Ca\(^{2+}\) provided by the EF-hands domains near ACTN4 leads to formation of the E3KARP-ACTN4 complex that initiates oligomerization. The evidence that ACTN4 is involved in NHE3 inhibition is based on transfection experiments with its ABDR14 domain, which appears to act as a dominant-negative mutant to inhibit Ca\(^{2+}\) regulation of NHE3. These cells express approximately equal amounts of ACTN4 and ABDR14 (Fig. 5A), and given that ABDR14 alone binds less well to E3KARP than full-length ACTN4 (Fig. 3), it would not be predicted that such a significant inhibition as demonstrated would occur. The explanation is not fully understood, but there are examples of other NHERF/E3KARP effects in which, despite the presence of large amounts of NHERF, its amount appeared to be rate-limiting in regulation. For instance, in the β2-receptor regulation of NHE3, transfecting NHERF into cells that contained endogenous NHERF increased the effect (39). We suggest that the pool of interacting molecules (E3KARP/ACTN4) is limited as the explanation for both the exaggeration of the effect by transfection of full-length ACTN4 and the dominant-negative effect of the ABDR14.

Endocytosis has been implicated previously (17, 18) in regulation of NHE3 activity by alteration of the amount of NHE3 localized in the plasma membrane. The intracellular localization of NHE3 in the recycling compartment was demonstrated both in nonepithelial cells, PS120 and AP-1 fibroblasts, and in epithelial cells, including Caco-2 and OK epithelial cells (17, 18, 30, 40). In all cells studied, NHE3 has been shown to cycle between the plasma membrane and the juxtanuclear compartment. In this context, we show that inhibition of NHE3 activity by elevation of Ca\(^{2+}\) involves internalization of NHE3 from the plasma membrane, and ACTN4 is involved in this endocytosis because overexpression of ABDR14 blocks the Ca\(^{2+}\)-dependent internalization (Fig. 6C).

Recently, a role for ACTN4 in endocytosis was proposed from the observation that ACTN4 exists in the macropinosome, which is formed during macropinocytosis and phagocytosis (41). Under basal conditions, ACTN4 bundles actin cytoskeleton, whereas it dissociates from actin with elevated Ca\(^{2+}\) (22). From these reports, one possible explanation for the role of ACTN4 in the Ca\(^{2+}\)-dependent regulation of NHE3 is that ACTN4 may physically link NHE3 to the actin cytoskeleton through E3KARP binding, and that actin cytoskeletal changes may be involved in formation of visible NHE3 clusters or in endocytosis of NHE3. Recent reports (42) showed that NHE3 associates with the actin cytoskeleton, and NHE3 activity can be modulated by changes in the actin cytoskeleton. From these results, we hypothesize that ACTN4 may regulate the Ca\(^{2+}\)-dependent endocytosis of NHE3 via linking NHE3 to the cytoskeleton.

In summary, the present study demonstrated that oligomerization and endocytosis of NHE3 following elevation of Ca\(^{2+}\) are necessary for inhibition of NHE3 activity induced by elevated [Ca\(^{2+}\)]. Ca\(^{2+}\)-dependent association between E3KARP and ACTN4 is required for both the oligomerization and endocytosis of NHE3. This is the first finding to show that oligomerization as well as endocytosis of NHE3 can be induced by protein-protein interaction regulated by intracellular signaling. However, all data to support this model came from the studies using PS120 fibroblasts exogenously transfected with NHE3, E3KARP, and ACTN4. Although the findings suggest a novel function of E3KARP, PS120 fibroblasts are not an optimal model system for the study of epithelial NHE3 regulation. Therefore, it will be important to demonstrate whether E3KARP and ACTN4 are also implicated in the Ca\(^{2+}\)-dependent regulation of NHE3 in epithelial cells as well as fibroblasts. In addition to NHE3, a variety of epithelial ion transporters, i.e. cystic fibrosis conductance regulator, sodium bicarbonate transporter, and sodium phosphate co-transporter, has been reported (43) to interact with NHERF and/or E3KARP. Considering the specific role of E3KARP in the Ca\(^{2+}\)-dependent endocytosis of NHE3, it will be interesting to assess the role of E3KARP in complex formation and/or endocytic regulation of other epithelial ion transporters and other E3KARP-binding proteins.

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