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Ca^{2+}-dependent inhibition of NHE3 requires PKCα which binds to E3KARP to decrease surface NHE3 containing plasma membrane complexes

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Lee-Kwon, Whaseon, Jae Ho Kim, Jung Woong Choi, Kazuya Kawano, Boyoung Cha, Darlene A. Dartt, Driss Zoukhri, and Mark Donowitz. Ca^{2+}-dependent inhibition of NHE3 requires PKCα which binds to E3KARP to decrease surface NHE3 containing plasma membrane complexes. *Am J Physiol Cell Physiol* 285: C1527–C1536, 2003. First published September 3, 2003; 10.1152/ajpcell.00017.2003.—The intestinal brush border (BB) Na^+/H^+ exchanger isoform 3 (NHE3) is acutely inhibited by elevation in the concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) by the cholinergic agonist carbachol and Ca^{2+} ionophores in a protein kinase C (PKC)-dependent manner. We previously showed that elevating $[Ca^{2+}]_i$ with ionomycin rapidly inhibited NHE3 activity and decreased the amount of NHE3 on the plasma membrane in a manner that depended on the presence of the PDZ domain-containing protein E3KARP (NHE3 kinase A regulatory protein, also called NHERF2). The current studies were performed in PS120 fibroblasts (NHE-null cell line) stably transfected with NHE3 and E3KARP to probe the mechanism of PKC involvement in Ca^{2+} regulation of NHE3. Pretreatment with the general PKC inhibitor, GF109203X prevented ionomycin inhibition of NHE3 without altering basal NHE3 activity. Similarly, the Ca^{2+}-mediated inhibition of NHE3 activity was blocked after pretreatment with the conventional PKC inhibitor Gö-6976 and a specific PKCα pseudosubstrate-derived inhibitor peptide. $[Ca^{2+}]_i$ elevation caused translocation of PKCα from cytosol to membrane. PKCα bound to the PDZ1 domain of GST-E3KARP in vitro in a Ca^{2+}-dependent manner. PKCα and E3KARP coimmunoprecipitated from cell lysates; this occurred to a lesser extent at basal $[Ca^{2+}]_i$ and was increased with ionomycin exposure. Biotinylation studies demonstrated that $[Ca^{2+}]_i$ elevation induced oligomerization of NHE3 in total lysates and decreased the amount of plasma membrane NHE3. Treatment with PKC inhibitors did not affect the oligomerization of NHE3 but did prevent the decrease in surface amount of NHE3. These results suggest that PKCα is not necessary for the Ca^{2+}-dependent formation of the NHE3 plasma membrane complex, although it is necessary for decreasing the membrane amounts of NHE3, probably by stimulating NHE3 endocytosis.

Na absorption; PDZ domains; signal complex

WE HAVE PREVIOUSLY DEMONSTRATED that elevation of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) rapidly inhibits the mammalian small intestinal neutral NaCl-absorptive process and its component brush border (BB) Na^+/H^+ exchanger NHE3 (7, 11–13, 33). Neutral NaCl absorption is important for digestive physiology, explaining small intestinal Na absorption between meals and the increase in the ileal Na absorption that occurs after meals. It is also important for the pathophysiology of diarrhea because it is the major Na-absorptive process inhibited in most diarrheal diseases. Elevating $[Ca^{2+}]_i$ by the cholinergic agonist carbachol and Ca^{2+} ionophores causes similar inhibition in ileal NaCl absorption (7, 12, 33). The inhibition of NHE3 activity by carbachol is associated with a decrease in the amount of BB NHE3, implicating changes in trafficking in its regulation by Ca^{2+} (Li XH and Donowitz M, unpublished observations). Protein kinase C (PKC) activity is necessary for the ileal inhibition of NaCl absorption caused by carbachol and Ca^{2+} ionophores and is associated with translocation to and activation of PKC in the ileal BB (7, 12, 33). However, it is still unknown how PKC plays a role in the Ca^{2+}-dependent inhibition of ileal NaCl absorption and which PKC isoform is involved.

It is becoming clear that kinase regulation of NHE3 requires physical association of NHE3 with multiple proteins, including PDZ domain proteins (18, 50). In previous studies to examine how elevated $[Ca^{2+}]_i$ regulates NHE3, we used a NHE-null cell model, the PS120 fibroblast, which is derived from Chinese hamster lung cells (30). These cells, in addition, lack the two-PDZ domain-containing protein E3KARP, which binds NHE3 and is required for elevated $[Ca^{2+}]_i$ inhibition of NHE3 (50). E3KARP or another related two-PDZ domain protein, Na^+/H^+ exchanger regulatory factor (NHERF; also called NHERF1), had previously been shown to be necessary to allow cAMP to regulate NHE3 in PS120 cells and in the renal proximal tubule OK cell line (50). E3KARP, bound to NHE3 at basal Ca^{2+}, forms a complex when Ca^{2+} is elevated that also includes α-actinin-4 (18). One explanation of the spec-

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ificity of E3KARP in the Ca^{2+} regulation of NHE3 was that E3KARP but not NHERF bound α-actinin-4 (18). The elevated Ca^{2+} inhibition of NHE3 appears to have two steps: *1*) formation of an NHE3/E3KARP/α-actinin-4 complex, and *2*) complex internalization, with complex formation preceding internalization (18).

The current studies used PS120 cells stably expressing NHE3 and E3KARP to demonstrate a role for PKC in these effects of elevated $[Ca^{2+}]_i$, to identify the PKC isoform involved, and to probe the mechanism by which PKC regulates NHE3. PKC is a family of homologous serine and threonine kinases. The eleven isozymes of PKC have been divided into three groups on the basis of their calcium and phospholipid requirements for activation: classic or conventional PKC (cPKC), including α, βI, βII, and γ, which are calcium and phospholipid dependent; new PKC (nPKC), including δ, ϵ, η, θ, and μ, which are calcium independent and phospholipid dependent, and atypical PKC (αPKC), including ξ and λ, which are calcium and phospholipid independent (28, 41). Fibroblasts contain at least α, δ, and ϵ isoforms (2, 37). In this study, PKCα is identified as the isoform involved in NHE3 regulation and its role shown to be in NHE3 complex internalization.

MATERIALS AND METHODS

Materials. pGEX4T-1 was from Amersham Pharmacia Biotech; ionomycin was from Sigma; NHS-SS-biotin and avidin-agarose were from Pierce; immobilized protein A-Sepharose beads were from Pharmacia Biotech. Monoclonal anti-vesicular stomatitis virus G protein (VSVG) antibody P5D4 was kindly provided by Dr. D. Louvard (Curie Institute, Paris, France). Monoclonal anti-E3KARP antibody was kindly provided by Dr. Pann-Ghill Suh (Pohang University of Science and Technology, Republic of Korea), and polyclonal anti-E3KARP antibody was provided by Dr. C. Yun (JHUSOM; current address: GI Division, Emory University School of Medicine, Atlanta, GA). Monoclonal anti-PKCα antibodies were from Transduction Laboratories. BL-21 cells were from Stratagene. PKC inhibitors, GF109203X, and Gö-6976 were from Biomol Research Laboratories, A synthetic peptide of a palmitylated pseudosubstrate sequence of PKCα (PALM-PKCα pseudosubstrate inhibitor [PALM-PKCα aa 15–28:K (PAM) DVANRFSRKGALRQ]) was provided by Darlene Dartt and Dris Zoukhri (Harvard Medical School, Cambridge, MA).

Cell culture. PS120/E3V/E3KARP (E3V refers to NHE3 epitope tagged on the COOH terminus with VSVG) has been previously described (17). Fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 25 mM $NaHCO_3$, 10 mM HEPES, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum in a 5% CO_2-95% O_2 humidified incubator at 37°C. Where appropriate, acid killing and hygromycin (600 units/ml) selections were applied to maintain high Na^+/H^+ exchange activity and expression of E3KARP, as described previously (21).

Measurement of Na^+/H^+ exchange. Na^+/H^+ exchange in PS120/E3V/E3KARP cells was determined fluorometrically using the intracellular pH-sensitive dye 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) with cells grown to 60–70% confluency on glass coverslips, as described previously, including consideration of buffering capacity (21). The cells were loaded with the acetoxymethyl ester of BCECF (BCECF-AM; 5 μM) in Na^+ medium (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 25 mM glucose, and 20 mM HEPES, pH 7.4) for 20 min and then washed with TMA^+ medium (130 mM tetramethylammonium chloride, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 25 mM glucose, and 20 mM HEPES, pH 7.4) to remove the extracellular dye; the coverslip was then mounted at an angle of 60° in a 100-μl fluorometer cuvette designed for perfusion and thermostated at 37°C. The cells were pulsed with 40 mM NH_4Cl in TMA^+ medium for 3 min, followed by TMA^+ medium, which resulted in the acidification of the cells. Na^+ medium was then added, which induced alkalinization of cells. Na^+/H^+ exchange (H^+ efflux) was calculated as described previously as the product of sodium-dependent change in intracellular pH (pH_i) times the buffering capacity at each pH_i. Kinetic analyses used a nonlinear regression data analysis program (Origin) that allowed fitting of data to a general allosteric model described by the Hill equation, with estimates for V_{max} and $K'[H^+]_i$ (21) and their respective distributions (SE). When GF109203X, Gö-6976, and PKC peptide inhibitors were studied, cells were pretreated with inhibitors for 15 min during the dye loading and during the prepulse period.

Glutathione S-transferase fusion proteins and binding assays. A series of glutathione *S*-transferase (GST) fusion proteins that include full-length NHERF and E3KARP and the various domains of E3KARP were prepared for this study, as described previously (18), by subcloning into the pGEX4T-1 vector. The GST-tagged fusion proteins, including the E3KARP first PDZ domain (P1, aa 12–92), the second PDZ domain (P2, aa 155–231), the COOH-terminal domain (C, aa 231–327), and the second PDZ domain plus the internal sequence between P1 and P2 (LnP2, aa 92–231), were expressed individually in *Escherichia coli* BL-21 cells. Purification of the GST fusion proteins was conducted as described previously with glutathione Sepharose (18).

In vitro binding of PKCα with GST-E3KARP fragments (2 μg each) was performed in *buffer A* (20 mM Tris·HCl, pH 7.4, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM EDTA, 150 mM NaCl, 0.1% Triton X-100). Free Ca^{2+} was set at 1 μM with 0.9 mM $CaCl_2$ added to *buffer A*. PKCα purified from baculovirus-infected Sf9 cells was kindly provided by Dr. Sung Ho Ryu (Pohang University of Science and Technology, South Korea). An aliquot (0.1 μg) of purified PKCα was added to *buffer A* and reacted with immobilized GST-E3KARP fragments in the presence of 1 μM free Ca^{2+} for 1 h at 4°C. The resultant beads were washed three times with *buffer A*, and the amounts of PKCα-bound to GST-E3KARP fragments were determined by Western blot analysis using anti-PKCα antibody.

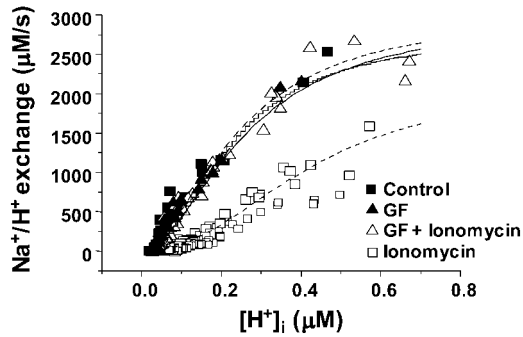
Immunoprecipitation and immunoblotting. Coimmunoprecipitation experiments were performed using cell lysates from PS120 cells expressing NHE3V and E3KARP in the absence or presence of ionomycin. Cell lysates were prepared in cell lysis buffer [20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, and 1% Triton X-100 with a mixture of protease inhibitors (Sigma) (nominally Ca^{2+} free)]. After a brief centrifugation for 5 min at 10, 000 *g*, the supernatants were subjected to protein assay. Aliquots of lysates (1 mg) were incubated with the anti-E3KARP antibody for 2 h at 4°C, followed by the addition of 35 μl of 50% protein A-Sepharose beads and gentle mixing on a rotator at 4°C overnight. After being washed five times with the wash buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, and 1% Triton X-100), the bound proteins were eluted and analyzed by SDS-PAGE and immunoblotted with the anti-PKCα or E3KARP antibodies.

Cell surface biotinylation. The surface amount of NHE3 was measured by cell surface biotinylation as described (18). PS120 cells grown in 10-cm petri dishes were treated with either agonist or vehicle at 37°C and then were rinsed extensively with phosphate-buffered saline (PBS) followed by borate buffer (154 mM NaCl, 7.2 mM KCl, 1.8 mM $CaCl_2$, and 10 mM H_3BO_3, pH 9.0) at 4°C. Cells then were incubated for 40 min with 1.5 mg of NHS-SS-biotin in borate buffer. Unbound NHS-SS-biotin was quenched with Tris buffer (120 mM NaCl and 20 mM Tris, pH 7.4; N^+ buffer). Cells were then rinsed with PBS and lysed in 1 ml of 150 mM NaCl, 3 mM KCl, 5 mM EDTA trisodium, 3 mM EGTA, 1% Triton X-100, and 60 mM HEPES, pH 7.4, and sonicated for 20 s. The lysates were agitated at 4°C for 30 min and spun to remove insoluble cell debris. Protein content in the supernatant was measured by the method of Bradford, and equal amounts of cell lysate were incubated with streptavidin-agarose at 4°C. The remaining supernatant was retained as the intracellular fraction. The streptavidin-agarose beads were washed repeatedly in N^+ buffer and boiled in Laemmli sample buffer. The total, surface, and intracellular fractions were resolved by SDS-PAGE with boiling/β-mercaptoethanol. Separated proteins were transferred to nitrocellulose membrane and probed with monoclonal anti-VSVG antibody P5D4 (hybridoma culture medium at 1:5 dilution). Bands were visualized using enhanced chemiluminescence and quantified using densitometric analysis, as described (18).

Membrane PKCα and phosphoconventional PKC. PS120/E3V/E3KARP cells were incubated with 2 μM ionomycin (in Na medium) and compared with solvent control. Cells were then harvested on ice. The cells were washed twice with cold PBS, resuspended in homogenization buffer [20 nM Tris·HCl, pH 8.0, 2 mM EDTA, 2 mM dithiothreitol, 1 mM vanadate, 50 mM NaF, 5 mM sodium pyrophosphate (NaPPi), and 10 mM β-glycerophosphate with protease inhibitors from Sigma], and sonicated three times for 5 s each and then centrifuged at 1,000 *g* for 10 min at 4°C. Supernatants were collected and centrifuged in a TL-100.3 rotor at 40,000 rpm for 40 min. The high-speed pellet was designated as the membrane fraction and was solubilized in homogenization buffer, whereas the supernatant was designated as the cytoplasmic fraction. Protein concentrations were determined according to the Bradford method using reagents from Bio-Rad. The membrane pellet was resuspended in an equal volume of homogenizing buffer. Proteins (20 μg) of total membrane and cytosol were mixed 1:5 with 5× Laemmli's buffer. Samples were boiled for 5 min and subjected to Western analysis with monoclonal anti-PKCα antibodies for immunodetection.

RESULTS

Elevated $[Ca^{2+}]_i$-induced NHE3 inhibition is PKC dependent. Previous studies have shown that elevation of $[Ca^{2+}]_i$ by ionomycin and thapsigargin in PS120/E3V/E3KARP cells inhibit NHE3 activity with similar magnitude (18). We examined whether PKC activity was necessary for the ionomycin-induced inhibition of NHE3. As shown in Fig. 1, exposure to ionomycin (2 μM) for 10 min decreased NHE3 transport activity by 30–40% by decreasing the V_{max} and increasing the $K'[H^+]_i$, as described (18). To demonstrate the involvement of PKC in the Ca^{2+}/NHE3 signaling pathway, NHE3 transporter activity was examined after pretreatment with the general PKC inhibitor, GF109203X (38) (Fig. 1). Pretreatment with GF109203X for 15 min



Control		GF	
Vmax:	2994 ± 244	Vmax:	3057 ± 340
K'[H+]i (μM):	0.26	K'[H+]i (μM):	0.24
GF and Ionomycin		**Ionomycin**	
Vmax:	2784 ± 125	Vmax:	2183 ± 594
K'[H+]i (μM):	0.22	K'[H+]i (μM):	0.43

Fig. 1. Ionomycin-induced inhibition of Na^+/H^+ exchanger (NHE3) activity is PKC dependent. PS120 cells were rendered quiescent by serum removal for at least 4 h, and ionomycin (2 μM) was added for 10 min before NHE3 activity was assayed fluorometrically (BCECF) as Na^+-dependent intracellular pH (pH_i) recovery after an acid load. Cell was exposed to GF109203X (2 μM) for 15 min before Ca^{2+} elevation. Elevation of Ca^{2+} by ionomycin inhibits NHE3 activity. Pretreatment with GF109203X prevented the ionomycin-induced inhibition of NHE3 activity, and GF109203X did not alter basal NHE3 activity. The data represent 1 example of 3 similar experiments.

prevented the ionomycin-induced inhibition of NHE3 V_{max}. Treatment with GF109203X alone had no effect on NHE3 activity. These results demonstrate that PKC is necessary for elevated $[Ca^{2+}]_i$ inhibition of NHE3 activity.

PKCα is the isoform involved in elevated $[Ca^{2+}]_i$-induced NHE3 inhibition. Which isoform(s) of PKC was involved in the ionomycin-induced inhibition of NHE3 activity was further characterized. Of the classes of PKC, only conventional isoforms are Ca^{2+} dependent. The effect of Gö-6976, a specific inhibitor of conventional PKCs (24), and a synthetic peptide of a palmitoylated peptide derived from the pseudosubstrate sequence of PKCα (PALM-PKCα pseudosubstrate inhibitor) (14, 35, 53–55), which is a PKCα inhibitor, were studied. NHE3 transport activity was measured after pretreatment with Gö-6976 and the PALM-PKCα pseudosubstrate inhibitor. As shown in Fig. 2*A*, pretreatment with 1 uM of Gö-6976 for 15 min prevented ionomycin-induced inhibition of NHE3 activity. The lack of ionomycin effect on $K'[H^+]_i$ in this series of studies is interpreted as experimental variability. Cells treated with Gö-6976 alone had no effect on NHE3 activity. Similar results were obtained with pretreatment with the PALM-PKCα pseudosubstrate inhibitor (100 nM) with no effect on basal Na^+/H^+ exchange but prevention of ionomycin-induced inhibition (Fig. 2*B*). All PKC inhibitors caused similar magnitude of reversal of $[Ca^{2+}]_i$ inhibition of NHE3 activity. These data, including the magnitude of inhibition of elevated $[Ca^{2+}]_i$ regulation of NHE3, indicate that

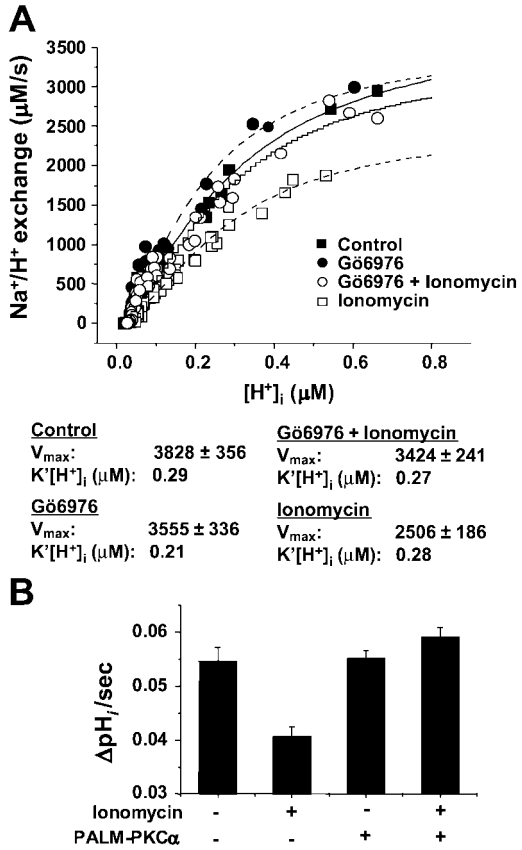


Fig. 2. PKCα is necessary for ionomycin-induced NHE3 inhibition. PS120 cells were pretreated (15 min) with the conventional PKC inhibitor Gö-6976 (*A*) and PALM-PKCα pseudosubstrate-derived inhibitor peptide (*B*). Both prevented the effect of ionomycin (2 μM) to inhibit NHE3. *A*: Gö-6976 (1 μM) prevented the ionomycin-induced inhibition of NHE3 activity without altering basal NHE3 activity. NHE3 activity is shown as the rate of Na^+-dependent cell pH recovery from an acid load. Results of 1 experiment that was repeated 3 times are shown. *B*: PALM-PKCα pseudosubstrate-derived inhibitor peptide (100 nM) prevented ionomycin inhibition without altering basal NHE3 activity. Initial Na/H exchange rates (dpH_i/s) are shown for the effect of ionomycin and PALM-PKCα pseudosubstrate-derived inhibitor peptide. Results are shown as means ± SE of 3 similar experiments.

PKCα is probably the single PKC isoform involved in Ca^{2+} inhibition of NHE3 activity.

PKCα binds to E3KARP in a Ca^{2+}-dependent manner. To understand how PKCα is involved in elevated $[Ca^{2+}]_i$ inhibition of NHE3, it was determined whether PKCα binds to either E3KARP (needed for Ca^{2+}-related NHE3 inhibition) or NHERF (not needed for Ca^{2+}-related NHE3 inhibition), and whether the binding is Ca^{2+} dependent. The interaction between NHERF or E3KARP and PKCα was examined by in vitro pulldown experiments using GST fusion proteins encoding full-length E3KARP and NHERF. Binding assays were performed in which GST fusion proteins of E3KARP and NHERF were incubated with an aliquot (0.1 μg) of purified PKCα (Fig. 3). In the absence of calcium, PKCα did not bind to GST or GST-NHERF but did bind to GST-E3KARP. The amount of PKC binding to GST-E3KARP was increased when free calcium was elevated to 1 μM. Note GST-NHERF also bound PKCα at 1 μM $[Ca^{2+}]_i$, but the binding was less than to E3KARP for the same amount of fusion protein. The data presented in Fig. 3 show that E3KARP binds PKCα and their interaction is increased by elevation in $[Ca^{2+}]_i$.

PKCα binds the PDZ1 domain of E3KARP. To determine which region of E3KARP interacts with PKCα, a series of GST fusion proteins that encode PDZ1, PDZ2, lnPDZ2, and COOH terminus of E3KARP were constructed (Fig. 4*A*). Assays in which GST fusion proteins were incubated with an aliquot (0.1 μg) of purified PKCα showed that the domain PKCα bound maximally was the PDZ1 domain of E3KARP, with much less binding to PDZ2. PKCα did not bind to the E3KARP COOH terminus or lnPDZ2 (Fig. 4*B*).

E3KARP coprecipitates PKCα in a Ca^{2+}-dependent manner. The previous experiments indicated that E3KARP bound to PKCα, predominantly via its PDZ1 domain. To determine whether this association occurs in intact cells, immunoprecipitation was performed, starting with PS120 cell lysate made from cells exposed to elevated $[Ca^{2+}]_i$/controls. As shown in Fig. 5, PKCα was coimmunoprecipitated with anti-E3KARP antibody. In addition, the in vivo results demonstrated that the association between PKCα and E3KARP was dependent upon elevation of $[Ca^{2+}]_i$. The amount of PKCα coprecipitated with E3KARP was increased by elevated $[Ca^{2+}]_i$ (Fig. 5). Thus the in vivo association of E3KARP and PKCα is calcium dependent, in agreement with the data presented in the in vitro binding assay (Fig. 3). Of note, the elevated Ca^{2+} conditions were only present in intact tissue exposed to ionomycin, and upon lysis, all tissues were in nominally Ca^{2+}-free conditions. This suggests that the NHE3/E3KARP complexes formed in vivo with elevated Ca^{2+} do not reverse rapidly in vitro when Ca^{2+} is lowered.

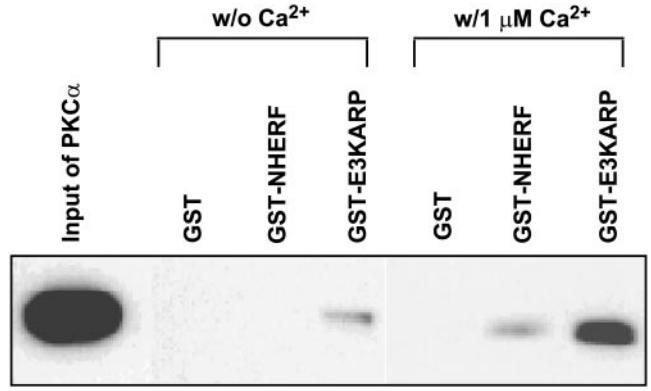


Fig. 3. PKCα binds E3KARP in vitro in a calcium-dependent manner. Full-length glutathione *S*-transferase (GST)-NHE3 kinase A regulatory protein (NHERF) and GST-E3KARP were immobilized on GST beads. An aliquot (0.1 μg) of recombinant PKCα was added to immobilized GST alone (*lanes 2* and *5*), GST-NHERF (*lanes 3* and *6*), and GST-E3KARP (*lanes 4* and *7*) in the absence or presence of 1 μM free calcium. The bound proteins were resolved by SDS-PAGE and transferred to immobilon membrane, and the amounts of PKCα were detected by Western blot analysis using anti-PKCα antibody. Input used for the experiment is shown in *lane 1*. Fusion protein (2 μg) was used. Lanes are numbered from left.

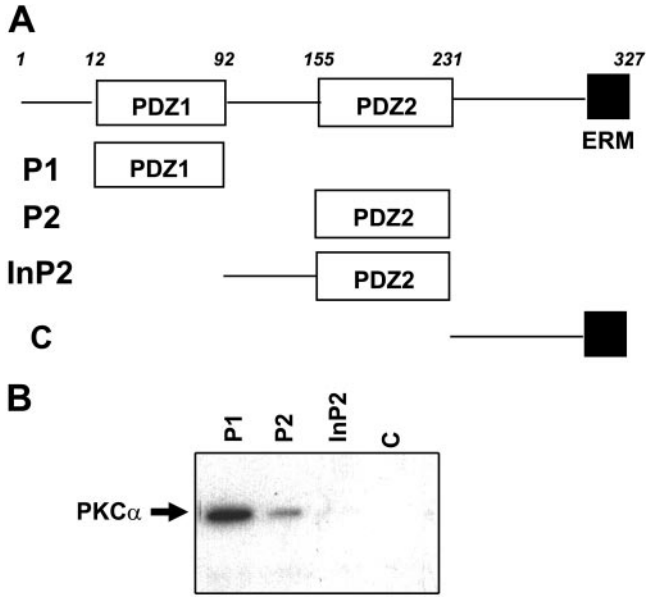


Fig. 4. PKCα binds to the PDZ1 domain of E3KARP. *A*: linear diagram of E3KARP shows the amino acids of the domains studied in *B*. *B*: series of immobilized GST fusion proteins (2 μg) that encoded different domains of E3KARP, including PDZ1, PDZ2, extended PDZ2 (LnP2), and the COOH terminus, were prepared. For the binding assays, aliquots (0.1 μg) of purified PKCα were added to immobilized GST fusion proteins and the bound proteins were resolved on SDS-PAGE. The amounts of PKCα-bound to GST-E3KARP domains were measured by Western blot analysis using anti-PKCα antibody.

Elevation of $[Ca^{2+}]_i$ induces subcellular redistribution of PKCα. To assess whether the elevation of $[Ca^{2+}]_i$ affected membrane PKC, as has been shown to occur with many examples of PKC activation (7, 45), the amounts of PKCα in membrane fractions were measured by Western analysis. Cells were treated with ionomycin for 2 min. Membrane and cytosol fractions were prepared by ultracentrifugation, and the amounts of PKCα were determined (Fig. 6*A*). Compared with unstimulated cells (Fig. 6*A*), treatment for 2 min with ionomycin increased membrane PKCα (Fig. 6, *A* and *B*). Although no loading control of another protein was simultaneously examined, that statistically significant changes occurred suggests that random differences in loading did not explain the finding. These data show that elevation of $[Ca^{2+}]_i$ induces a rapid increase of membrane PKCα amount in PS120/E3V/E3KARP cells.

Decrease in the amount of surface NHE3 by $[Ca^{2+}]_i$ elevation is PKCα dependent, but NHE3 oligomerization is PKC independent. Ca^{2+} inhibition of NHE3 in PS120/E3V/E3KARP is thought to involve two steps; complex formation and decrease in surface NHE3 (18). To determine which step or steps were PKC dependent, we used cell surface biotinylation. PS120/E3V/E3KARP cells were exposed to elevated $[Ca^{2+}]_i$ for 10 min with and without pretreatment (15 min) with two PKC inhibitors, GF109203X and Gö-6976. Cells were then placed at 4°C, and cell surface biotinylation was performed with NHS-SS-biotin and total and surface

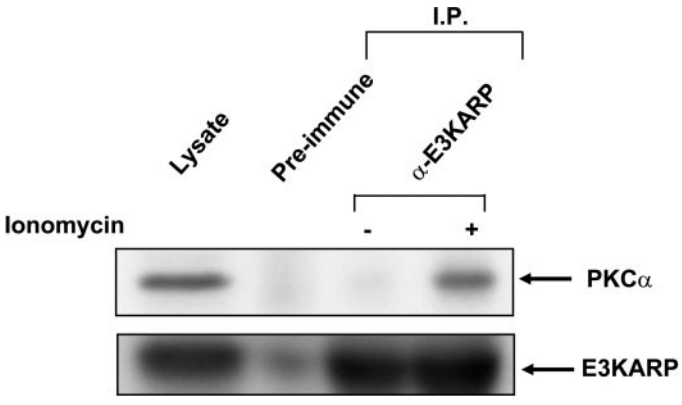


Fig. 5. Elevation of $[Ca^{2+}]_i$ increases the coprecipitation of PKCα by E3KARP. PS120/E3KARP/E3V cells were treated with ionomycin (2 μM)/control for 10 min, lysed, and immunoprecipitated (IP) with anti-E3KARP antibody or preimmune serum. The immune complexes were resolved by SDS-PAGE, and the precipitated proteins were probed with anti-PKCα or anti-E3KARP antibodies, the latter to show the extent of immunoprecipitation. Preimmune serum was only exposed to lysates from ionomycin exposed cells. A representative study, which was repeated 3 times with similar results, is shown.

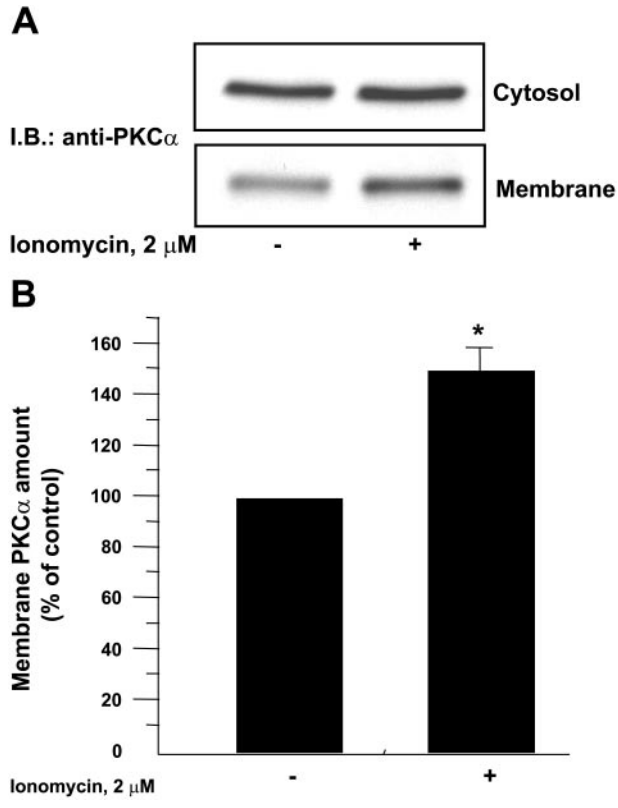


Fig. 6. Elevation of $[Ca^{2+}]_i$ redistributes PKCα to membranes of PS120/E3V/E3KARP cells. Cells were treated with ionomycin (2 μM) or vehicle at 37°C for 2 min and then separated into membrane and cytosol. *A*: PKCα amount was determined with anti-PKCα antibody after 2 min of ionomycin (2 μM) exposure in vitro. Membrane and cytosol fractions were resolved by SDS-PAGE and probed with anti-PKCα antibody. A representative experiment is shown. *B*: quantification by scanning densitometry for the membrane fraction in *A* normalized to the nonionomycin control. Data are means ± SE of 3 experiments. $^*P < 0.05$ compared with control (ionomycin).

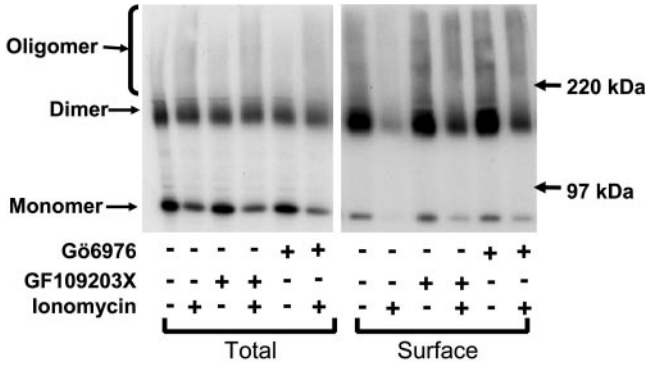


Fig. 7. Pretreatment with PKC inhibitors does not alter distribution of NHE3 among oligomer, dimer, and monomer but prevents the decrease in surface amount of NHE3. Ionomycin (2 μM, 10 min, 37°C) or vehicle-pretreated cells, some pretreated with GF109203X (2 μM, 15 min) or Gö-6976 (1 μM, 15 min), were surface labeled with biotin after temperature was lowered to 4°C. Surface proteins were retrieved from the cell by streptavidin precipitation. NHE3 protein abundance was quantitated by scanning densitometry of Western blots using NHE3 immunoblotted with anti-vesicular stomatitis virus G protein (VSVG). Total (*left*) and surface (*right*) NHE3 were examined. This is a representative experiment that was repeated 3 times with similar results. Molecular mass standards are shown at *right*.

NHE3 was determined (Fig. 7). Total NHE3 (Fig. 7, *left*) is used to illustrate the effect of the PKC inhibitors on NHE3 oligomerization. Under basal conditions, NHE3 is mostly in monomers and a size approximately double that of the monomer (dimers), but with elevated Ca^{2+} there is less NHE3 monomer and more dimer and larger sizes. Under basal conditions total NHE3 is distributed: monomer, 40 ± 10%,; dimer, 55 ± 82; oligomers, 5 ± 7%. After Ca^{2+} elevation, NHE3 is distributed: monomer 25 ± 7%; dimer, 60 ± 10%; oligomers 15 ± 10%. The dimers and oligomers are assumed to represent only NHE3 but could represent NHE3 in larger complexes with other proteins. There was no change in this Ca^{2+} related "oligomerization" of total NHE3 by pretreatment with either PKC inhibitor when the same amount of protein was used for immunoprecipitation.

Surface NHE3 is shown in Fig. 7, *right*. Ca^{2+} elevated for 10 min was associated with a decrease in surface NHE3 as reported (18) (surface NHE3 15 ± 6% of total under basal condition and 5 ± 10% of total after Ca^{2+} elevation). The PKC inhibitors largely prevented the Ca^{2+}-induced decrease in surface amount of NHE3 (surface NHE3 after GF 11 ± 8% of total and after 60 min 13 ± 7% of total, both of which were not significantly different from the basal levels). Thus PKC is involved in NHE3 internalization and not in NHE3 complex formation. The greater Ca^{2+}-induced decrease in the amount of surface NHE3 than the decrease in transport (compare Figs. 1 and 2) in this study differs from our previous report (18) in which the magnitude of the changes was more similar. Possible explanations as to why there are differences between surface NHE3 and amount and activity include the possibility that the molecules of NHE3 have different activities based on the complex in which they are present. This is totally speculative for Ca^{2+} regulation, although this was suggested for NHE3 in renal proximal tubule brush border on the basis of megalin association (5, 6).

DISCUSSION

These studies demonstrate a role for PKCα in elevated $[Ca^{2+}]_i$ regulation of NHE3. We previously reported that PKC was an intermediate in elevated Ca^{2+}-induced inhibition of ileal neutral NaCl absorption and BB Na^+/H^+ exchange (12, 30, 41). In this ileal model, elevated $[Ca^{2+}]_i$ was associated with translocation of PKC amount and activity to the BB, and inhibiting PKC with the PKC inhibitor H7 prevented elevated $[Ca^{2+}]_i$ from decreasing neutral NaCl absorption (7). The current studies in PS120 fibroblasts demonstrated similar Ca^{2+}-dependent inhibition of NHE3, which was PKC dependent. This is consistent with similar regulation being involved in ileal Na absorptive cells, which contain endogenous NHE3 and E3KARP (50), and in PS120 fibroblasts, which express only exogenous E3KARP and NHE3 (50). The fact that E3KARP is necessary for this Ca^{2+} regulation of NHE3 has been demonstrated in PS120 cells. The advances in the studies presented here in PS120 cells are mechanistic, although the mechanisms have not yet been shown to apply to the BB of intestinal Na-absorptive epithelial cells.

A single PKC isoform is probably involved in Ca^{2+} regulation of NHE3 on the basis of similarity in inhibition of the Ca^{2+} effect on NHE3 activity of the general PKCα GF109203X inhibitor and the specific PKCα inhibitor. The PKC isoform involved in NHE3 regulation was PKCα based on pharmacological (inhibitor) and biochemical studies. The Ca^{2+} inhibition was reversed at least 70% by the general PKC inhibitor GF109203X, the conventional PKC inhibitor Gö-6976, and a palmitoylated pseudosubstrate-derived PKCα inhibitor peptide, which is specific for PKCα in intact cell studies (14, 35, 52–55). The GF109203X data indicated that PKC was involved. Because a Ca^{2+}-dependent effect was being studied, it appeared likely that a conventional PKC would be involved because only the conventional class of PKCs are Ca^{2+} dependent (28, 41). Note, the specific PKC isoforms that are present in PS120 fibroblasts have not been defined. However, the PKC isoforms in two other fibroblast lines (AP-1 cells, a Chinese hamster ovarian cell derivative, and NIH 3T3 cells) were shown to include PKCα, δ, and ϵ (2, 37). Thus the only conventional PKC isoform likely to be involved in Ca^{2+} regulation of NHE3 in fibroblasts was the α isoform. The involvement of PKCα was supported by inhibition of Ca^{2+} effects on NHE3 by the conventional PKC inhibitor Gö-6976. The involvement of PKCα was strongly suggested by the use of the palmitoylated PKCα peptide inhibitor. These pharmacological results were supported by demonstration of an increased amount of membrane PKCα that occurred at the earliest time point studied (2 min) in the PS120 cells after $[Ca^{2+}]_i$ elevation.

The fact that PKCα was the involved isoform was used to begin further understanding mechanisms of NHE3 regulation by elevated $[Ca^{2+}]_i$. PKCα is the only conventional PKC isoform to have a PDZ binding domain on its COOH terminus (QSAV) (30). Thus we postulated that PKCα might bind to E3KARP and with elevated $[Ca^{2+}]_i$, PKCα might enter the E3KARP-NHE3-containing complex that we previously showed also includes α-actinin-4 (18). The present studies showed that with elevated Ca^{2+}, there was increased coprecipitation of PKCα by E3KARP. There was some basal association of E3KARP with PKCα with increased in vivo coprecipitation of PKCα by E3KARP in the presence of 1 μM $[Ca^{2+}]_i$.

Because we showed that in the absence of E3KARP no increased NHE3 oligomerization occurred (18), we suggest E3KARP is central to elevated $[Ca^{2+}]_i$ NHE3 complex formation. The fact that PKCα binds to PDZ1 of E3KARP but not PDZ2, whereas NHE3 and α-actinin-4 (the latter previously shown to be involved in Ca^{2+} inhibition of NHE3 and to be in an NHE3/E3KARP complex) bind to PDZ2 and not PDZ1 (18, 49), indicates that PKCα and either NHE3 or α-actinin-4 could associate with the same E3KARP molecule. It is possible that E3KARP multimerizes either with other E3KARP molecules or with other PDZ domain-containing proteins in the ileal BB to include NHE3, α-actinin-4, and PKCα in the same complex, if not on the same E3KARP molecule. Further studies will be required to define the stoichiometry of the components of this complex and to identify which other proteins are in this Ca^{2+}-dependent NHE3, E3KARP, α-actinin-4, PKCα-containing complex. Given that the size of the NHE3-containing complexes, as determined by sucrose density gradient centrifugation, on the surface of NHE3 containing PS120, Caco-2, and OK cells is ~400 to ~900 kDa under basal $[Ca^{2+}]_i$ conditions and that the size of NHE3 complexes increase with elevated $[Ca^{2+}]_i$ (Ref. 3 and Li X and Donowitz M, unpublished), the full picture of which proteins form this complex with elevated $[Ca^{2+}]_i$ remains incomplete.

We previously reported that E3KARP is necessary for Ca^{2+} inhibition of NHE3, whereas NHERF is not able to play this role (18). This specificity of E3KARP appears to be due to the fact that E3KARP is necessary for forming the NHE3-containing complexes by binding to α-actinin-4, which we showed is necessary for Ca^{2+} inhibition of NHE3. In contrast, specificity does not clearly relate to E3KARP binding to PKCα. As shown in Fig. 3, at basal $[Ca^{2+}]_i$ only E3KARP and not NHERF binds PKCα, whereas at 1 μM $[Ca^{2+}]_i$ both E3KARP and NHERF bind PKCα, although E3KARP binds a larger amount of PKCα than NHERF. Whether intracellular conditions are such that with elevated $[Ca^{2+}]_i$, PKCα binds NHERF in addition to E3KARP is not known. Thus understanding of the specificity of the E3KARP/PKCα interaction in NHE3 regulation compared with that of the other BB PDZ domain-containing proteins remains incomplete but is unlikely to be a major factor.

The role of PKCα in NHE3 regulation has been partially defined in these studies. Elevated $[Ca^{2+}]_i$ inhibits NHE3 by affecting two steps: *1*) formation of plasma membrane NHE3-containing complexes in which NHE3 appears to multimerize, and *2*) internalization. Elevated $[Ca^{2+}]_i$ decreases the amount of surface and increases the amount of intracellular NHE3, an effect prevented by PKC inhibitors (Fig. 7). The step PKCα is involved in does not appear to be complex formation. Rather, it is in the decrease in surface NHE3. What this PKC-dependent step consists of is not yet established but is likely to be due to increased endocytosis, with decreased exocytosis possible but less likely. The rapid time course is consistent with the fact that at least part of this effect involves increased endocytosis. PKC has previously been shown to stimulate endocytosis, and less commonly to stimulate exocytosis, with the α and ϵ isoforms most commonly involved (4, 10, 16, 20, 25, 27, 32, 36, 46). The forms of PKC stimulated endocytosis includes clathrin-dependent, clathrin-independent, and fluid-phase endocytosis (3, 10, 32, 36). PKC affects endocytosis of multiple plasma membrane proteins, including receptors and transport proteins, although not all plasma membrane proteins are affected. Moreover, PKC has been shown to phosphorylate multiple components of the endocytosis machinery (8, 9, 23, 31, 34). In previous studies, phorbol esters also affected endocytosis via non-PKC-dependent mechanisms, making it necessary to consider more direct demonstration of a PKC role in aspects of endocytosis (1). In addition, regulation of NHE3 by endocytosis in response particularly to elevated cAMP has previously been elegantly demonstrated (17b). The new observation here is that elevated Ca^{2+} acting by PKC stimulates endocytosis of an NHE3 complex that includes E3KARP, α-actinin-4, and PKCα and is dependent on E3KARP, α-actinin-4, and PKCα (18). Whether NHE3 or other complex components undergo PKC-dependent endocytosis that exceeds that of other endocytosed plasma membrane proteins will be of interest to determine. This is especially true compared with other E3KARP-binding proteins, which are not part of this complex, which must be defined to understand how specifically Ca^{2+} regulates Na absorption in these cells.

The substrate PKC phosphorylates to allow the decrease in surface NHE3 has also not been determined. Although NHE3 contains PKC phosphorylation consensus sequences, it has not yet been clearly shown to have increased phosphorylation of these sites with elevated $[Ca^{2+}]_i$. Certainly, the role of an increase in NHE3 phosphorylation in Ca^{2+} inhibition/endocytosis of NHE3 is not established. Moe et al. (44) previously showed that phorbol ester exposure increases NHE3 phosphorylation in AP-1 fibroblasts. However, there was no correlation between changes in phosphorylation of NHE3 and whether a specific clone of NHE3 responded to phorbol ester with elevated, decreased, or nonchanged NHE3 activity (44). AP-1 cells do not express E3KARP (Ref. 48 and Donowitz M, Cha B, and Yun C, unpublished observations). In addition, in non-

E3KARP-containing PS120/E3V cells, phorbol ester did not alter NHE3 phosphorylation, determined by two-dimensional phosphopeptide mapping (47). Whether the phosphorylation status of NHE3 in response to elevated $[Ca^{2+}]_i$ is involved with its regulation when E3KARP is present is unknown. Because PKC also phosphorylates multiple components of the endocytosis machinery, including dynamin, synaptojanin, AP2, and clathrin (8, 9, 23, 31, 39), and the fact that there are putative PKC phosphorylation sites in other components of the NHE3 complex, including α-actinin-4 and E3KARP (36), the substrate(s) PKCα phosphorylates that is necessary for NHE3 internalization remains unknown. The fact that PKC can stimulate endocytosis independently of phosphorylation of the protein taken up has been emphasized recently (1).

The nature of the NHE3 complexes, including what appears to be NHE3 multimers under basal conditions, which increase with elevated Ca^{2+}, is not fully understood. Fafournoux, Noel, and Pouyssegur showed in 1994 (15) that NHE1 and NHE3 appeared as both monomers and dimers on SDS/reducing gels. The amount of monomer was decreased and that of dimer increased with crosslinkers. This initially suggested that NHEs, including NHE3, might exist as complexes or multimers under basal conditions. Our results for NHE3 under basal conditions are similar, although with a larger percent of total NHE3 appearing as dimers and oligomers (monomer 40 ± 6%; dimer 55 ± 8%; oligomer 5 ± 7%, $n = 3$). In addition, NHE3 has been shown to scaffold several Ca^{2+}-binding proteins, including calmodulin, calcineurin homologous protein, and megalin (5, 6, 22, 29, 39, 40, 46), further suggesting that it could be involved in Ca^{2+}-related complex formation. Nonetheless, in spite of seeing what appears to be NHE3 complex formation by confocal microscopy (18) and by surface biotinylation studies, the nature of the complexes are uncertain and attempts to isolate the complexes are underway. Definition of the meaning of NHE3 multimerization is complicated by the fact that boiling samples in Laemmli's solution before running the samples on a gel causes changes similar to the Ca^{2+} effect, decreasing monomeric and increasing dimeric and multimeric NHE3 (data not shown). Nonetheless, the fact that when all samples were similarly boiled, Ca^{2+}-exposed tissue still has decreased monomeric and increased dimeric and multimeric NHE3 is most consistent with Ca^{2+} exposure, causing NHE3 complex formation.

NHE3-containing complexes scaffolded by E3KARP or NHERF have now been reported for cAMP and Ca^{2+} regulation of NHE3 (15, 19, 42, 43). The complexes involved in cAMP regulation of NHE3 in fibroblasts and OK cells (opossum kidney proximal tubule cell line) appear to accept either NHERF or E3KARP, seem static over time, and also involve ezrin, PKAII, and actin. In OK cells, although over time cAMP leads to a decrease in surface NHE3 (17b, 51), there have been no other reported changes in the nature of the NHE3-E3KARP-containing plasma membrane complexes. The complexes involved in Ca^{2+} regulation are different in that they are dynamic with α-actinin-4 and PKCα joining the complex with elevated Ca^{2+}, and all complex components appear together in the cytosol over time with Ca^{2+} elevation (Ref. 18 and data not shown). An interesting, unresolved question is whether the complexes involving E3KARP and NHE3 are the same in both cAMP and Ca^{2+} regulation of NHE3. In the past, cAMP and Ca^{2+} regulation of NHE3 have been shown to be at least additive in PS120 cells (Cha BY, Yun C, and Donowitz M, unpublished observations). We hypothesize that NHE3 spontaneously exists in separate complexes that take part in different aspects of its regulation.

Regulation of NHE3 by elevated $[Ca^{2+}]_i$ almost certainly occurs by multiple, time-dependent changes in trafficking and intracellular handling of NHE3. Until now, only the initial complex formation and internalization have been demonstrated, although multiple molecules come together for that regulation. Changes in rates of NHE3 exocytosis, half-life, distribution in lysosomes/proteosomes, and binding to regulatory proteins in the NHE3/E3KARP complex are all predicted to be part of regulation of NHE3 activity. Defining which of these processes are affected by PKCα and its short- and long-term role in NHE3 regulation will be important to understand Ca^{2+} regulation of NHE3 in normal physiology and disease.

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DISCLOSURES

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