Phospholipase C-γ Binds Directly to the Na+/H+ Exchanger 3 and Is Required for Calcium Regulation of Exchange Activity*

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Multiple studies suggest that phospholipase C-γ (PLC-γ) contributes to regulation of sodium/hydrogen exchanger 3 (NHE3) in the small intestine, although the mechanism(s) for this regulation remain unknown. We demonstrate here that PLC-γ binds directly to the C terminus of NHE3 and exists in similar sized multiprotein complexes as NHE3. This binding is dynamic and decreases with elevated [Ca2++]i. The PLC-γ-binding site in NHE3 was identified (amino acids 586–605) and shown to be a critical regulatory domain for protein complex formation, because when it is mutated, NHE3 binding to PLC-γ as well as NHERF2 is lost. An inhibitory peptide, which binds to the Src homology 2 domains contained in PLC-γ without interrupting binding of PLC-γ to NHE3, was used to probe a non-lipase-dependent role of PLC-γ. In the presence of this peptide, carbachol-stimulated calcium inhibition of NHE3 was lost. These results mirror previous studies with the transient receptor potential channel and suggest that PLC-γ may play a common role in regulating the cell-surface expression of ion transporters.

The sodium/hydrogen exchanger (NHE)² gene family member NHE3 (Slc9a3) plays an integral role in neutral sodium absorption in the mammalian intestine (1). NHE3 activity is altered during normal digestion and is inhibited in diarrheal diseases (2, 3). The latter remains a major worldwide health problem. NHE3 exists in large multiprotein complexes (4, 5) and is regulated through multiple protein–protein interactions involving its C terminus (aa 455–832) (6). For example, the C terminus of NHE3 directly binds proteins, which include calpain homologous protein, NHERF1–4, ezrin, casein kinase 2 (CK2), and megalin (7–12). Some of these proteins mediate regulation of NHE3 activity by forming multiprotein complexes that bring together specific signal transduction proteins to facilitate phosphorylation, endocytosis, and exocytosis of NHE3 (7–9, 11).

Elevated levels of intracellular calcium [Ca2++]i, inhibit electroneutral sodium absorption and NHE3 in the intact rabbit small intestine (13–16). Decreased NHE3 activity is due, in part, to decreased surface expression of NHE3 and is associated with increased size of multiprotein NHE3-containing complexes (5). In the rabbit ileum, carbachol elevates [Ca2++]i, via basolateral membrane-localized muscarinic acetylcholine receptors (M3) and inhibits NHE3 activity by ~40% (13, 14, 17). The carbachol-induced increase in [Ca2++]i occurs initially at the apical membrane, followed by increased intracellular calcium throughout the cytoplasm at later time points (18). This increases brush border (BB) levels of diacylglycerol, protein kinase C (PKCo), and activated brush border phospholipase D (19). Moreover, carbachol causes a rapid translocation of activated (but not tyrosine-phosphorylated) phospholipase C-γ1 (PLC-γ) to the apical membrane (19). Although previous studies have suggested a role for PLC in elevated [Ca2++]i, inhibition of NHE3, a molecular mechanism for this effect has not been defined.

Through its phospholipase catalytic domains, PLC-γ hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate two second messengers as follows: (i) diacylglycerol, which destabilizes lipid membranes and directly activates PKC, and (ii) inositol 1,4,5-trisphosphate, which signals the release of calcium from intracellular stores (20). In addition, PLC-γ has other signaling properties that are lipase-independent and act through its other protein–protein interacting domains (e.g. split pleckstrin homology domains, PH-n and PH-c; and Src homology domains, SH2 and SH3) (21–23). For example, the PH-n domain of PLC-γ directly binds β-tubulin and modulates microtubule assembly, whereas the SH3 domain acts as a guanine nucleotide exchange factor for the phosphatidylinositol-3-OH kinase enhancer as well as dynamin-1 (24–26). In addition, the PLC-γ PH-c domain directly binds the transient receptor potential channel (TRPC3) calcium channel, regulating surface expression and agonist-induced calcium entry (27). This regulation was recently shown to be dependent on direct binding of PLC-γ SH2 and PH-c domains with the transcription factor TFII-I (27, 28).
**PLC-γ Directly Binds NHE3**

We now demonstrate that PLC-γ and NHE3 exist in large protein complexes and directly bind via amino acids 586–605 of NHE3 and the PH-c domain in PLC-γ. The direct binding of PLC-γ and NHE3 is dynamic, decreasing with elevated [Ca²⁺]. Finally, using a peptide that selectively binds to the SH2 domains of PLC-γ, we show that PLC-γ is required for calcium-mediated inhibition of NHE3 activity.

**EXPERIMENTAL PROCEDURES**

Reagents—4-Bromo-A23187, a nonfluorescent analog of the calcium ionophore A23187, was from Biomol (29). Carbachol was from Sigma. BODIPY 577/618 maleimide was from Invitrogen.

Antibodies—Affinity-purified mouse monoclonal antibody to human PLC-γ was from Millipore. Mouse monoclonal anticalcium vesicular stomatitis virus (VSV)-G protein antibody P5D4 (hybridoma culture medium) was from Drs. T. Kreiss and D. Louvard. Monoclonal anti-HA antibody was from Covance.

Fusion Proteins—Fusion proteins of full-length rabbit NHE3 C terminus (aa 475–832) and the C terminus of NHE3 divided into four fragments (F1 (aa 475–588), F2 (aa 589–667), F3 (aa 668–747), and F4 (aa 748–832)) were generated as His₆-tagged fusion proteins, as described previously (30). Briefly, NHE3 C-terminal cDNA inserts were subcloned into pET30a vector (Novagen). His-tagged fusion proteins were generated after transformation into Escherichia coli and subsequently purified with Ni²⁺-nitrilotriacetic acid resin per the manufacturer’s protocol (Qiagen).

Yeast Two-hybrid—Experiments were performed with the Matchmaker 3 yeast two-hybrid system (Clontech), following the manufacturer’s instructions.

Pulldown Assays—Cell lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.8, 1% Triton X-100, 1 mM EDTA) was added to 100 μg of His₆-NHE3 purified fragments. GST-Sepharose beads and purified GST-PLC-γ PH-c were incubated on a rotator for 1 h at 4 °C, washed three times with lysis buffer, and quenched with 20 μl of SDS sample buffer. Co-precipitates were resolved by SDS-PAGE and analyzed by Western blot analysis.

Cell Lines—PS120 fibroblasts lack all endogenous plasma membrane NHE3s, NHERF1 (minimal expression), NHERF2, NHERF3, and NHERF4. These cells, when stably expressing rabbit NHE3 with a C-terminal VSV-G protein epitope tag and NHERF2, are called PS120/NHE3/NHERF2 cells, as described previously (4). Briefly, NHE3 C-terminal cDNA inserts were subcloned into pET30a vector (Novagen). His-tagged fusion proteins were generated after transformation into Escherichia coli and subsequently purified with Ni²⁺-nitrilotriacetic acid resin per the manufacturer’s protocol (Qiagen).

**TABLE 1**

| NHE3 cells | Amino acids | Mutation(s) |
|------------|-------------|-------------|
| NHE3-690   | 1–832       |             |
| NHE3-605   | 1–832       |             |
| NHE3-585   | 1–585       |             |
| NHE3-509   | 1–509       |             |
| NHE3-H633A | 1–832       | H633A       |
| NHE3-F2Δ7  | 1–832       | R588A, E589A, D597A, E602A, R604A, R605A, R606A |

Caco-2BBe cells express all four members of the NHERF gene family and small amounts of NHE3. Triple HA-tagged rabbit NHE3 was expressed by adeno virus into Caco-2BBe cells for transport and biochemical analysis. Caco-2BBe cells were grown on transwell filters (Corning Glass) until post-confluent for 12 days in Dulbecco’s modified Eagle’s medium supplemented with 25 mM NaHCO₃, 10 mM HEPES, 0.1 mM nonessential amino acids, 50 units/ml penicillin, 50 μg/ml streptomycin, and 50% bovine serum in a 5% CO₂, 95% O₂ incubator at 37 °C. Cells were serum-starved overnight and then treated with 6 mM EGTA for 2 h at 37 °C. Caco-2BBe cells were then exposed to 3HA-NHE3 adenovirus for 6 h at 37 °C. Cells were allowed to recover in normal media over the next 40 h before study.

Co-immunoprecipitation—PLC-γ or NHE3 was immuno-precipitated (IP) from the total lysate of PS120/NHE3/NHERF2 and Caco-2BBe/3HA-NHE3 cells (in the presence of 1% Triton X-100). All IPs were done at 4 °C with constant mixing on a rotary shaker. Briefly, each sample (1 mg of total cell lysate per IP) was first precleared with either protein A-Sepharose beads (Sigma) or protein A beads conjugated to rabbit anti-mouse secondary antibody for 1 h. The precleared lysate was then incubated with 4 μg of antibodies to PLC-γ, NHE3 (VSV-G or HA), or preimmune serum (control) for 1 h. Protein A-Sepharose beads were then added to each IP mixture, and incubation was continued for another 1 h. The beads were washed four times with phosphate-buffered saline buffer containing 0.1% Tween 20 (Sigma). The IP pellets were analyzed by SDS-PAGE and Western blotted with corresponding antibodies.

Caco-2BBe cells were grown on 10-cm² transwell Petri dishes until post-confluent for 12 days. On day 12 post-confluency, cells were serum-starved for 4 h and then treated with 6 mM EGTA for 2.5 h. Cells were then incubated with the adenovirus 3HA-NHE3 construct for 6 h in serum-free media. After infection, cells were allowed to recover in normal media for 40 h prior to carbachol treatment. On day 14 post-confluency, cells were serum-starved again for 4 h and treated either with vehicle or 10 μM carbachol (Sigma) for 10 min at 37 °C. Adenovirus-infected Caco-2BBe cells were washed three times in ice-cold phosphate-buffered saline containing 50 mM Tris. Cells were collected and lysed in 500 μl of ice-cold lysis buffer (10 mM HEPES, 50 mM NaCl, 5 mM EDTA, 1 mM benzamidine, 0.5% Triton X-100). Cell lysate was solubilized for 30 min at 4 °C with end-over-end rotation and subsequently homogenized 10 times using a 23-gauge needle. Cellular debris was cleared by centrifugation at 14,000 rpm for 15 min. Supernatant was incubated with either anti-HA affinity matrix (Roche Applied Science) or anti-VSV-G-agarose beads (Sigma) for 2 h with end-over-end rotation at 4 °C. Samples were washed five times with...
lysis buffer, and immunoprecipitated proteins were eluted from beads with 2× sample buffer. Samples were resolved by 10% SDS-PAGE, and proteins were detected with anti-HA and anti-PLC-γ antibodies and visualized on an Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE).

Sucrose Density Gradient Centrifugation—PS120/NHE3/NHERF2 cells were grown to confluency in 10-cm Petri dishes, and Caco-2BBe cells were grown to post-confluency in 12 days on Transwell filters in 10-cm Petri dishes, infected with adenovirus NHE3 construct, and studied on day 14. Cells were solubilized in 1 ml of N+ buffer (50 mM HEPES, Tris, pH 7.4, 150 mM NaCl, 3 mM KCl, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1 mM pepstatin, 1 mM iodoacetamide, 1% Triton X-100), sonicated, and spun briefly to remove unbroken cells, nuclei, and cell debris. Solubilized cell extracts (4 °C) were applied to the top of discontinuous 2.5–30% sucrose gradients (increasing at increments of 2.5% sucrose containing 0.1% Triton X-100). After centrifugation for 16.5 h at 4 °C at 150,000 g, the gradients were fractionated (20 fractions; 0.6 ml each) from the bottom with a perfusion pump, and NHE3 and PLC-γ expression was visualized by Western blot with anti-VSV-G monoclonal antibody (for NHE3 in PS120 cells), anti-HA monoclonal antibody (for NHE3 in Caco-2BBe cells), and anti-PLC-γ monoclonal antibody, as described above. NHE3 and PLC-γ protein expression was detected with IRdye700 and IRdye800 anti-mouse secondary antibodies (Rockland) using the Odyssey Infrared Imaging System (Li-Cor).

BODIPY Peptides—Peptide synthesis and purification were performed by the Synthesis and Sequencing Facility at The Johns Hopkins University School of Medicine. Coupling of the peptide to BODIPY 577/618 maleimide was performed according to the manufacturer’s protocol (Invitrogen). Excitation/ emission spectral analysis of BODIPY-conjugated peptides demonstrated base-line fluorescent levels at 530 nm, which is the wavelength used in measurement of NHE3 activity described below (Fig. 5 C).

Measurement of Na+/H+ Exchange—Cellular Na+/H+ exchange activity in PS120 cells grown to ~70% confluency on glass coverslips was determined fluorometrically using the intracellular pH-sensitive dye, 2′,7′-bis(carboxyethyl)5–6-carboxyfluorescein-acetoxyethyl ester (BCECF-AM, 5 μM; Molecular Probes, Eugene, OR), as described previously (32). PS120 cells were exposed to 40 mM NH4Cl alone or with 4-bromo-A23187 (0.5 μM) during a 15-min dye loading, as described previously (32). Cells were perfused initially with TMA+ solution (130 mM tetramethylammonium chloride, 5 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 1 mM NaH2PO4, 25 mM glucose, 20 mM HEPES, pH 7.4), before being switched to Na+ solution (130 mM NaCl instead of tetramethylammonium chloride) for the Na+-dependent pHi recovery. At the end of each experiment, the fluorescence ratio was calibrated to pHi using the high potassium/nigericin method. Na+/H+ exchange activity data were calculated as the product of Na+ -dependent changes in pHi times the buffering capacity at each pHi, and individual points shown in the figures are rates of Na+/H+ exchange calculated at multiple pHi, values using at least three coverslips per condition in a single experiment. Kinetics of Na+/H+ exchange were analyzed by Hill plot using Origin (Microcal Software) to estimate Vmax and K(H+), in individual experiments. Means ± S.E. were determined from at least three separate experiments (32).

Statistics—Results were expressed as mean ± S.E. Statistical evaluation was by analysis of variance or Student’s t test.

RESULTS

NHE3 and PLC-γ Co-localize in Multiprotein Complexes—NHE3 exists in large protein complexes, and these complexes change as part of acute regulation (5). For example, carbachol exposure increases the size of NHE3 complexes in ileal brush border (5). Complexes formed in PS120 cells with elevated [Ca2+]i, include NHE3, NHERF2, α-actinin-4, and activated PKCa (9) and are similar to those found in ileal brush border membranes after carbachol exposure (5). These findings suggest the involvement of a conserved regulatory mechanism in the response of NHE3 to elevated [Ca2+]i. Because activated PLC-γ appears to reside at the apical membrane of intestinal epithelial cells near NHE3, we asked whether PLC-γ associates with NHE3 in similar multiprotein complexes. In both PS120 fibroblasts stably expressing NHERF2 and VSV-G-tagged
NHE3 (PS120/NHE3/NHERF2) and Caco-2BBe, polarized intestinal epithelial cells transiently infected with 3HA-NHE3, NHE3, and PLC-γ/H9253 exist in similar sized multiprotein complexes (ranging in size from ~200 to 1200 kDa) as determined by sucrose density gradient centrifugation (n = 3; Fig. 1, and see “Experimental Procedures”). Furthermore, we also determined in PS120/NHE3/NHERF2 cells that a portion of NHE3 and PLC-γ/H9253 reside in lipid rafts containing caveolin (data not shown). Results from these experiments are consistent with a direct interaction between NHE3 and PLC-γ; therefore, we expanded our binding studies using co-precipitation and in vitro approaches.

To test the hypothesis that NHE3 and PLC-γ associate in vivo, co-immunoprecipitation studies were performed (n = 6). Total cell lysates were prepared from PS120/NHE3/NHERF2 cells and from Caco-2BBe/3HA-NHE3 cells. NHE3 was immunoprecipitated and subjected to SDS-PAGE for Western blot analysis for PLC-γ interactions. In both PS120 cells and Caco-2BBe cells, immunoprecipitated NHE3 co-precipitates PLC-γ (Fig. 2, A and B). Additionally, this association was dynamic, being abolished in Caco-2BBe/3HA-NHE3 cells treated with 10 μM carbachol for 10 min (Fig. 2B).

Previous studies have demonstrated that the C terminus of NHE3 binds multiple proteins that appear to be involved in signal transduction and cytoskeletal attachment (7–12). Many of these studies utilized in vitro binding techniques with fragments of the NHE3 C terminus (amino acids 455–832) to establish protein-protein interactions. Similarly, in this study, we used purified His₆-tagged protein fragments (F1–F4) of the NHE3 C terminus to isolate the region(s) responsible for the direct interaction of NHE3 and PLC-γ (Fig. 3A). Previous findings demonstrated that the PH-c domain of PLC-γ bound to TRPC3 (27); therefore, we tested whether the PH-c domain could bind to NHE3 as well. His₆-tagged fusion proteins of NHE3 C-terminal fragments were bacterially expressed, purified, and tested for binding against the purified GST-tagged PLC-γ PH-c domain (amino acids 865–920) (Fig. 3B; see “Experimental Procedures”). In this in vitro assay GST-PLC-γ PH-c bound the F2 fragment (aa 589–667) of NHE3 but not the other C-terminal fragments. Similar results were obtained in a yeast two-hybrid binding assay, wherein the F2 fragment of NHE3 bound the PH-c domain of PLC-γ but not the PH-n, SH2, or SH3 domains (Fig. 3C). Based on these results, we conclude that the NHE3 F2 fragment binds directly to the PH-c domain of PLC-γ.

NHE3 and PLC-γ Co-immunoprecipitate and Bind Directly in Vitro and Cell Culture Models—To extend these findings and map the interaction site in NHE3, we conducted co-immunoprecipitation studies with full-length PLC-γ and overexpressed C-terminal deletion mutants of NHE3 in PS120 cells (Fig. 4A). Epitope-tagged
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FIGURE 4. NHE3 deletion mutants define PLC-γ-binding site. A, co-immunoprecipitation of NHE3 and PLC-γ from PS120/NHERF2 cells stably expressing various NHE3 (VSV-G-tagged) truncation mutants. Truncation of NHE3 C terminus to aa 605 still observed in vivo association with PLC-γ as determined by immunoblot. Binding of PLC-γ was abolished in NHE3-585 and NHE3-509 (data not shown) truncation mutants, suggesting that aa 586–605 of NHE3 are necessary for PLC-γ binding. HA, anti-HA negative control. Similar results were obtained from three independent experiments. B, co-immunoprecipitation studies using NHE3-F2Δ7 mutant. Seven charged amino acid residues between aa 585 and 606 mutated to alanines completely abolished the in vivo association of PLC-γ and NHE3. PLC-γ-binding was still present in the NHE3-H633A mutant. Similar results were obtained from three independent experiments. IB, immunoblot. C, NHE3 activity was measured (using BCECF) in PS120/NHERF2 cells stably transfected with either wild type NHE3 or NHE3-F2Δ7 mutant. The F2Δ7 mutant exhibited significantly reduced basal NHE3 activity compared with wild type NHE3. In addition, [Ca\(^{2+}\)]\(_i\) inhibition of NHE3 activity (via A23187) was abolished in PS120/NHE3-F2Δ7/NHERF2 cells. n = 4 for each condition. Rate of NHE3 activity (V\(_{\text{max}}\)) expressed as mean ± S.E. NS mean not significant.

(VSV-G) NHE3 proteins were immunoprecipitated (using anti-VSV-G antibody; see “Experimental Procedures”) and subjected to SDS-PAGE for Western blot analysis for PLC-γ interactions. An anti-HA antibody served as a negative control because NHE3 was not HA-tagged in PS120 cells. These experiments revealed that amino acids 586–605 of NHE3 are required for association with PLC-γ. This was a surprising result given that previous experiments from our laboratory have assigned this region as an NHERF-2 and CK2-binding site (10, 30). We generated a full-length NHE3 construct mutated in the seven charged residues in this region (NHE3-F2Δ7, phospholipase-binding mutant) and performed the analysis again (Fig. 4B). This mutant was not able to co-precipitate. NHE3-F2Δ7 was also not able to bind NHERF2, an interaction that is necessary for calcium regulation of NHE3 (9) (data not shown). NHE3-F2Δ7 activity was assessed using the pH-sensitive dye BCECF, and intracellular calcium levels were elevated by treating PS120 cells with 4-bromo-A23187 (Fig. 4C, n = 4) as well as the calcium-mobilizing agent thapsigargin (data not shown).

Compared with wild type preparations, NHE3-F2Δ7 displays markedly reduced basal activity (~2-fold) and no calcium-mediated inhibition. Because we demonstrated that PLC-γ binds to NHE3 between aa 589 and 667, we chose another area outside of the region that is necessary for binding (aa 586–605) to serve as a negative control. Previous studies by van Rossum et al. (27) demonstrated that the region necessary for binding PLC-γ to TRPC3 shared some homology with amino acids 630–634 of NHE3. Therefore, we generated a mutant (H633A) of this region and found that the association of NHE3 and PLC-γ was not altered (Fig. 4B). These results suggest that this area (aa 630–634) is not necessary for direct binding of NHE3 and PLC-γ. Taken together, these data suggest that NHE3 aa 586–605 is a critical hub for multiple regulatory protein-protein interactions. However, the overlapping interaction sites of NHERF2 and PLC-γ within NHE3 make it difficult to elucidate a specific functional role for PLC-γ binding with loss-of-binding mutations. Therefore, we sought an independent way to evaluate this association.

PLC-γ SH2 Domains Contribute to Basal NHE3 Activity and Are Necessary for Calcium Regulation—Previously Caraveo et al. (28) identified an amino acid sequence within the transcription factor, TFII-I, that directly bound the SH2 domains of PLC-γ. Furthermore, phosphorylation of a tyrosine residue within the peptide sequence (termed here “active” peptide) was necessary for the interaction with PLC-γ (28). We reasoned the following: (i) the SH2 domains of PLC-γ may be functionally important in the NHE3 complex, and (ii) if so, these same peptides might afford a way to modulate and assess the functional interactions between PLC-γ and NHE3 without loss-of-binding mutants. If correct, this would imply that basal and/or calcium regulation of NHE3 activity requires PLC-γ to serve as a scaffold and/or regulate specific proteins via its SH2 domain.

To test this hypothesis, we utilized the active peptide described above and a nonphosphorylated “inactive” peptide as a negative control (Fig. 5A). These peptides were conjugated with a fluorescent hydrophobic BODIPY molecule that rendered the peptides cell-permeable (33) as demonstrated by our immunofluorescent images (Fig. 5B). Importantly, only conjugated BODIPY molecules are fluorescent at the emission wavelength studied (617 nm) as demonstrated by emission scans in Fig. 5C. We then incubated PS120/NHE3/NHERF2 cells with either the BODIPY-conjugated active or inactive peptides and tested whether PLC-γ binding to NHE3 was altered by co-immunoprecipitation experiments in PS120 cells (Fig. 5D). Neither peptide interfered with the ability of PLC-γ to bind NHE3.

NHE3 activity in PS120 cells was assessed in the presence of either the active or inactive peptides under basal and elevated calcium conditions. In PS120 cells stably expressing NHE3 and NHERF2, exposure to the active peptide resulted in ~30% decrease in basal NHE3 activity compared with untreated control preparations (Fig. 6A, n = 6). Conversely, basal NHE3 activity was not significantly different from untreated controls when exposed to the inactive peptide. Next we investigated whether the SH2 domains play a role in calcium inhibition of NHE3 activity. PS120 cells were either exposed to vehicle or 4-bromo-A23187 (0.5 μM) in the presence and absence of either...
the inactive or active BODIPY-conjugated peptides. In the untreated control, elevated intracellular calcium caused a significant decrease in NHE3 activity compared with basal activity (Fig. 6A, n = 6). This effect was similar in cells exposed to the inactive peptide. However, in cells incubated with the active peptide, calcium regulation of NHE3 activity was abolished. Taken together, these results suggest that the SH2 domains of PLC-γ play a functional role in basal and calcium regulation of NHE3 activity in PS120 fibroblasts.

To compare these findings to effects in an epithelial cell, we repeated the above experiments in the polarized intestinal epithelial cell line, Caco-2BBe, which expresses endogenous NHE1 and NHE2 but very small amounts of NHE3 (1). Therefore, we measured sodium/hydrogen exchange in the presence of 50 μM HOE-694, which is known to completely inhibit activity of endogenous NHE1 and NHE2 but does not affect NHE3 (34, 35). Caco-2BBe cells were grown on filters until post-confluent for 12 days and were then infected with an adenovirus 3HA-NHE3 construct (see “Experimental Procedures”). NHE3 activity was determined 48 h after the infection. As above, Caco-2BBe/3HA-NHE3 cells were exposed to either BODIPY-conjugated inactive or active peptides to determine whether basal and/or calcium regulation of NHE3 activity involves the SH2 domains of PLC-γ. Unlike results obtained in PS120 cells, the active peptide did not significantly alter basal NHE3 activity. Nevertheless, the
results of these experiments demonstrate that the SH2 domain of PLC-γ is necessary for calcium regulation of NHE3 activity.

DISCUSSION

The results of this study are the first to demonstrate that NHE3 and PLC-γ directly bind, that the interaction is dynamic, decreasing with elevated \([Ca^{2+}]\), and that this association plays a significant role in regulation of NHE3 activity. Previous studies by our laboratories in intact rabbit ileum have suggested a potential role for PLC-γ in calcium regulation of NHE3 activity (19). Carbachol treatment resulted in a transient increase of \([Ca^{2+}]\), first at the apical membrane, followed by a general rise in \([Ca^{2+}]\). In addition, there was a rapid increase in BB PLC-γ expression and activity, which was not tyrosine-phosphorylated, following carbachol treatment that also was associated with an increase in BB diacylglycerol levels and PKC activity. Moreover, the increase in BB PLC-γ is not only due to increased \([Ca^{2+}]\), as this effect was not observed in rabbit ileum treated with A23187 (19). Although the results of these studies suggested a role for PLC-γ in NHE3 regulation, this study was designed to determine whether a direct relationship exists between NHE3 and PLC-γ.

NHE3 exists in large protein complexes that range in size from 200 to 1200 kDa, and these complexes include NHE3 binding partners known to be involved in acute regulation of NHE3 activity (e.g. PKCa, α-actinin-4, CK2, and NHERF2). We showed that NHE3 and PLC-γ exist in similar sized multiprotein complexes under basal conditions in both PS120 fibroblasts and Caco-2BBe cells as determined by sucrose density centrifugation. NHE3 immunoprecipitated PLC-γ in both PS120 and Caco-2BBe cells supporting that NHE3 and PLC-γ associate in cell culture models.

We demonstrated that the PH-c domain of PLC-γ directly binds the NHE3 C terminus between aa 589 and 667. We have previously demonstrated that this region of the NHE3 C terminus binds several proteins, which include CK2 and NHERF1–4. The PH-c domain of PLC-γ has been demonstrated to bind both TRPC3 and the transcription factor TFII-I (27, 28). In these studies, the direct binding of the PH-c domain of PLC-γ was associated with non-lipase-dependent regulation of agonist-induced calcium entry. Given these results and the fact that the PH-c domain of PLC-γ directly binds NHE3, we reasoned that PLC-γ may exert lipase-independent regulation on NHE3 activity as well.

Using peptide sequences generated by Caraveo et al. (28), which specifically bound the SH2 domains of PLC-γ, we tested whether the SH2 domains of PLC-γ played a role in NHE3 regulation. In both PS120 and Caco-2BBe cells exposed to the active peptide, elevation of \([Ca^{2+}]\), by either carbachol or A23187 failed to inhibit NHE3 activity, whereas \([Ca^{2+}]\) inhibition occurred with the inactive peptide control. These results suggest that PLC-γ SH2 domains bind unidentified signaling proteins necessary for \([Ca^{2+}]\)-mediated inhibition of NHE3 activity. Moreover, these results imply a scaffolding role for PLC-γ in mediating elevated \([Ca^{2+}]\), regulation of NHE3 activity.

Previous studies in Caco-2BBe cells and intact rabbit ileum have demonstrated carbachol-increased BB diacylglycerol levels and PKC activity that are consistent with lipase-dependent functions of PLC-γ (19). These data along with the results from this study suggest that the role of PLC-γ in \([Ca^{2+}]\), regulation of NHE3 activity may involve both lipase-dependent and lipase-independent functions. Whether the PLC-γ involved is still associated with NHE3 or is released dynamically by carbachol is not known.

In addition to \([Ca^{2+}]\), regulation of NHE3 activity, the PLC-γ SH2 domains are involved in regulating basal NHE3 activity in PS120 cells. These results indicate that PLC-γ probably scaffolds other signaling proteins to NHE3 under basal conditions. Moreover, this association seems to be dynamic in response to...
elevated $[Ca^{2+}]$, in that PLC-γ may scaffold one protein under basal conditions and another under elevated $[Ca^{2+}]$, conditions. However, because blocking PLC-γ SH2 domains did not affect basal NHE3 activity in Caco-2BBe cells, PLC-γ regulation of basal NHE3 activity in the intestine remains uncertain.

NHE3 is known to exist in multiple pools within intestinal epithelial cells (5). It is possible that the nature of NHE3 association in different membrane microdomains (e.g. lipid rafts) could explain our differing results. For example, we have observed in PS120 cells that NHE3 and PLC-γ co-localize in caveolin-positive lipid rafts.3 Caco-2BBe cells, on the other hand, do not express caveolin, although NHE3 endocytosis also occurs after elevation of $[Ca^{2+}]$, in these cells. Future studies will be required to resolve whether such differences can explain the unique role of the PLC-γ SH2 domains in regulating basal NHE3 activity in fibroblasts.

Although the results of this study suggest that PLC-γ may exert lipase-independent regulation of NHE3 activity, the mechanisms of this regulation remain to be determined. The results of our study showed that PLC-γ directly binds the NHE3 C terminus and modulates calcium regulation of NHE3 activity by a mechanism in which the SH2 domain(s) of PLC-γ is needed. Because SH2 domains are protein-protein interacting domains, PLC-γ may link other signaling proteins to NHE3 that could regulate NHE3 activity. For example, PLC-γ may link kinases and/or phosphatases to NHE3-containing complexes to regulate NHE3 activity by altering the phosphorylation state of NHE3 or of other molecules that bind and regulate NHE3. Also, other proteins linked to the SH2 domains of PLC-γ could be brought into NHE3-containing complexes in a dynamic manner by changes in signaling. Additionally, PLC-γ and NHE3 partially co-localize to lipid raft containing microdomains, and thus it is possible that NHE3 activity is correlated to its presence or absence in lipid rafts. Because we have demonstrated that the association of NHE3 and PLC-γ is dynamic in response to elevated $[Ca^{2+}]$, (by carbachol), it would be interesting to determine whether NHE3 expression in lipid rafts is altered after carbachol treatment. In conclusion, this study is the first to demonstrate that NHE3 and PLC-γ bind directly and that this association plays a significant role in regulation of NHE3 activity.

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