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Elevated Intracellular Calcium Stimulates NHE3 Activity by an IKEPP (NHERF4) Dependent Mechanism

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Key Words
NHE3 • PDZ domain • IKEPP • Exocytosis • Intracellular Ca^{2+}

Abstract
The ileal brush border (BB) contains four evolutionarily related multi-PDZ domain proteins including NHERF1, NHERF2, PDZK1 (NHERF3) and IKEPP (NHERF4). Why multiple related PDZ proteins are in a similar location in the same cell is unknown. However, some specificity in regulation of NHE3 activity has been identified. For example, elevated intracellular Ca^{2+} ($[Ca^{2+}]_i$) inhibition of NHE3 is reconstituted by NHERF2 but not NHERF1, and involves the formation of large NHE3 complexes. To further evaluate the specificity of the NHERF family in calcium regulation of NHE3 activity, the current study determined whether the four PDZ domain containing protein IKEPP reconstitutes elevated $[Ca^{2+}]_i$ regulation of NHE3. In vitro, IKEPP bound to the F2 region (aa 590-667) of NHE3 in overlay assays, which is the same region where NHERF1 and NHERF2 bind. PS120 cells lack endogenous NHE3 and IKEPP. Treatment of PS120/NHE3/IKEPP cells (stably transfected with NHE3 and IKEPP) with the Ca^{2+} ionophore, 4-Br-A23187 (0.5μM), stimulated NHE3 V_{max} activity by ~40%. This was associated with an increase in plasma membrane expression of NHE3 by a similar amount. NHE3 activity and surface expression were unaffected by A23187 in PS120/NHE3 cells lacking IKEPP. Based on sucrose density gradient centrifugation, IKEPP was also shown to exist in large complexes, some of which overlap in size with NHE3, and the size of both NHE3 and IKEPP complexes decreased in parallel after $[Ca^{2+}]_i$ elevation. FRET experiments on fixed cells demonstrated that IKEPP and NHE3 directly associated at an intracellular site. Elevating $[Ca^{2+}]_i$ decreased this intracellular NHE3 and IKEPP association. In summary: (1) In the presence of IKEPP, elevated $[Ca^{2+}]_i$ stimulates NHE3 activity. This was associated with increased expression of NHE3 in the plasma membrane as well as a shift to smaller sizes of NHE3 and IKEPP containing complexes. (2) IKEPP directly binds NHE3 at its F2 C-terminal domain and directly associates with NHE3 *in vivo* (FRET). (3) Elevated $[Ca^{2+}]_i$ decreased the association of IKEPP and NHE3 in an intracellular compartment. Based on which NHERF family member is expressed in PS120 cells, elevated $[Ca^{2+}]_i$ stimulates (IKEPP), inhibits

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(NHERF2) or does not affect (NHERF1) NHE3 activity. This demonstrates that regulation of NHE3 depends on the nature of the NHERF family member associating with NHE3 and the accompanying NHE3 complexes.

Introduction

In normal digestive physiology, the BB Na^+/H^+ exchanger, NHE3, mediates the majority of the NaCl and $NaHCO_3$ absorption in the ileum [1]. Acute stimulation and inhibition of NHE3 both occur as part of digestive physiology. Short-term regulation of NHE3 activity is achieved through a variety of factors which affect NHE3 surface expression, phosphorylation, as well as interactions with the cytoskeleton and accessory proteins, including the PDZ domain containing proteins, NHERF1 and NHERF2 [1, 2].

The NHERF (Na^+/H^+ Exchanger Regulatory Factor) family of PDZ proteins consists of four evolutionarily related members, all of which are expressed in epithelial cells of the mammalian small intestine [2]. NHERF1 and NHERF2 have been previously shown to be necessary for some aspects of acute NHE3 regulation [3-10]. Recently, two other PDZ domain containing proteins, PDZK1 (NHERF3) and IKEPP (NHERF4), have been identified and shown to have sequence homology with NHERF1 and NHERF2 [11-14]. However, unlike NHERF1 and NHERF2, which are comprised of two tandem PDZ domains flanked by a C-terminal ezrin/radixin/moesin (ERM) binding domain, PDZK1 and IKEPP consist of only four PDZ domains and have no additional protein-protein interacting domains.

IKEPP was initially identified by yeast-two hybrid screening of a human intestinal cDNA library using the C-terminus of the intestinal BB guanylyl cyclase C (GCC) as bait [13]. IKEPP is expressed in epithelial cells in the human small intestine, colon and kidney and is localized to the BB and to a cytoplasmic region adjacent to the apical membrane in Caco-2 cells [13]. Although IKEPP has been shown to bind apical membrane proteins, including the type IIa sodium/phosphate co-transporter (NaPi IIa) [15] and the multi-drug resistance related protein 2 (MRP2) [16], the role of IKEPP in the regulation of these proteins has not yet been elucidated. However, in COS7 cells transiently expressing IKEPP and guanylate cyclase C (GCC; receptor for guanylin), addition of the *E. coli* heat stable enterotoxin, STa, increased cGMP synthesis significantly less compared to cells expressing GCC lacking its C-terminal PDZ binding domain [13]. While the results of this study suggested a role for IKEPP in the inhibition of stimulated GCC activity, the mechanism of this regulation remains unknown.

Several physiological and pathophysiological agonists, acting through $[Ca^{2+}]_i$-induced second messenger systems, are known to inhibit electroneutral NaCl absorption in the small intestine [1, 17]. Elevation of $[Ca^{2+}]_i$ has previously been demonstrated to inhibit NHE3 activity in a NHERF2, but not NHERF1, dependent manner [5]. NHERF2 regulation of NHE3 involves the formation of multi-protein complexes that include NHE3, NHERF2, α-actinin-4, and PKCα which induces endocytic removal of NHE3 from the plasma membrane [5, 18]. Since multiple PDZ proteins exist in the apical pole of epithelial cells, the current study was designed to determine whether IKEPP also reconstitutes Ca^{2+} regulation of NHE3 activity. A simple cell system was selected for study initially to allow definition of the role of NHERF4 in NHE3 regulation separate from interactions involving the multiple other NHERF proteins.

Materials and Methods

Reagents

4-bromo-A23187, the non-fluorescent analog of the calcium ionophore, A23187, was from Biomol [19].

Antibodies

Affinity-purified mouse monoclonal antibody against human IKEPP was generated at the UNC Immunology core facility using hexahistidine tagged(His_6)-IKEPP. Briefly, full-length, human IKEPP was expressed in SF-9 insect cells infected with IKEPP baculovirus. Viruses were generated using the Invitrogen FastBac system (Invitrogen, Carlsbad, CA). Details of the infection and culture conditions have been previously described [20]. Mice were immunized with His_6-IKEPP purified using Ni-NTA sepharose and mono-Q columns. The hybridoma line, UNC8.16, was selected for production and the epitope was mapped to amino acid residues 1-50. The antibody was purified from culture supernatants using protein G sepharose.

Rabbit polyclonal antibody to NHE3 (Ab1381) was previously characterized by Hoogerwerf et al. [21]. Mouse monoclonal anti-vesicular stomatitis virus (VSV)-G antibody P5D4 (hybridoma culture medium) was provided by Drs. T. Kreiss and D. Louvard. Mouse monoclonal anti-(VSV)-G Cy3-conjugated antibody was from Sigma. Anti-hemagglutinin (HA) Alexa Fluor 488 conjugate and goat anti-mouse IgG Alexa Fluor 568 secondary antibody were from Invitrogen.

Immunofluorescence

PS120/NHE3/pcDNA3.1 and PS120/NHE3/IKEPP cells were seeded on glass coverslips and grown to 70% confluence. Cells were serum starved for 3 hours and then treated with vehicle or the non-fluorescent calcium ionophore, 4-bromo-A23187 (0.5ìM) for 15 minutes. Cells were washed three times in phosphate-buffered saline (PBS) and fixed for 15 min with 3% paraformaldehyde (PFA) in PBS. The fixed cells were washed with PBS buffer and treated with 20mM L-Glycine for 10 minutes. Cells were placed in blocking solution (PBS containing 15% fetal bovine serum, 2% BSA and 0.1% saponin) for 45 minutes at room temperature. Primary antibodies were incubated for 1 h at room temperature in blocking solution at the following dilutions: 1:100 for monoclonal anti-(VSV)-G Cy-3-conjugated antibody (anti-NHE3 antibody) and 1:100 for monoclonal anti-IKEPP antibody. Cells were then washed three times with PBS and incubated with anti-mouse Alexa-fluor 488 conjugated secondary antibodies (1:100) for 1 hour at room temperature. Cells were washed three times with PBS and mounted with Gel Mount (Sigma) and then examined with a Zeiss LSM510 confocal fluorescence microscope.

Caco-2BBe cells were seeded onto 25mm 0.02µm Anapore filters (Nunc) and grown until 12 days post-confluency. On day 12, Caco-2BBe cells were treated with 6mM EGTA for 2 hours prior to infection with 3HA-NHE3 adenovirus construct. After 40 hours, cells were washed with ice-cold PBS and fixed for 30 minutes at 4°C with 3% paraformaldehyde in PBS. Cells were blocked and permeabilized with 1% bovine serum albumin (BSA) and 0.075% saponin in PBS for 1 hour at 4°C. Caco-2BBe cells were incubated with primary antibodies to HA tag on NHE3 (1:100) and endogenous IKEPP (1:100) for 1 hour at room temperature. IKEPP expression was detected using goat anti-mouse Alexa Fluor 568 secondary antibody. Cells were washed with 0.1% BSA and 0.075% saponin in PBS prior to mounting. Membrane inserts were detached from wells, placed on glass microscope sliders (Fisher Scientific), mounted then examined with a Zeiss LSM510 confocal fluorescence microscope.

Intact distal ileum was obtained from wild-type C57Bl/6 mice, fixed in 3.5% paraformaldehyde (PFA) in PBS at 4°C and paraffin-embedded. Histologic sections (4 µm thick) were mounted onto Superfrost microscope slides (Fisher Scientific Co., Arlington, VA) and heat fixed. Slides were microwaved for antigen recovery in 10 mM sodium citrate buffer, pH 6 (Sigma Chemical Company, St. Louis, MO) at power level setting 9 (Panasonic Model# NN-C980B Conventional Microwave Oven, Secaucus, NJ), for 2-5 min. After cooling for 30 min, sections were washed in PBS and preblocked with 5% normal goat serum (NGS) in PBS for 30 min at room temperature. Sections were then incubated 48 h (4°C) with a monoclonal IKEPP antibody diluted 1:100 in 5% NGS, or with rabbit polyclonal 1381 antibody to NHE3 (anti-GST-C-terminal 85a.a. of NHE3) diluted 1:100 in 5% NGS-PBS. Ileal sections were then washed twice in PBS for 10 min and incubated with anti-mouse Alexa-fluor 488 (IKEPP) and anti-rabbit Alexa-fluor 568 (NHE3) secondary antibodies, each diluted 1:100 for 1 hour at room temperature. Nuclei were counterstained with Hoescht 33342. Sections were washed twice with PBS, autofluorescence quenched with 1% Sudan Black (Sigma) in 70% methanol for 10 minutes at room temperature, and mounted with Gel Mount. Ileal sections were imaged using a Zeiss LSM/510 confocal fluorescence microscope using a 63X water immersion lens.

Fusion Proteins

Fusion proteins of full-length rabbit NHE3 and individual fragments of the C-terminus of NHE3 (F1 (aa 475–589), F2 (aa 590–667), F3 (aa 668–747), and F4 (aa 748–832)) were generated as described previously [22]. Briefly, NHE3 C-terminal cDNA inserts were subcloned into pET30a vector (Novagen). His_6-tagged fusion proteins were generated after transformation into *E. coli* and subsequently purified with Ni^{2+}-nitrilotriacetic acid resin per manufacturer's protocol (Qiagen).

Protein-Protein Interactions

Protein overlay (far Western) assays was used to examine the interaction of recombinant NHE3 C-terminus, (full-length and fragments 1-4), on blots with recombinant IKEPP (overlay). Recombinant NHE3 proteins (3 µg) were transferred to an Immobilon PVDF membrane (Millipore) and blocked overnight with 10% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST). The membrane was incubated for two hours with *in vitro* translated ^{35}S-methionine/cysteine IKEPP made using a TNT Lysate-Coupled Transcription/Translation kit (Promega). Following incubation, the membrane was washed with TBST and exposed to a phophorimager screen. Bands were visualized using a STORM-840 PhosphorImager and processed using ImageQuant software.

Co-immunoprecipitation

NHE3 was immunoprecipitated from the total lysate of PS120 cells stably expressing NHE3 and IKEPP (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 was first precleared with Protein-A-Sepharose beads (Sigma) for 1 h. The precleared lysate was then incubated with 4 µg of antibodies to NHE3 or preimmune serum (control) for 1 h. Protein A-Sepharose B beads were then added to each IP mixture and incubation was continued for another 1 h. The beads were washed 4 times with PBS buffer containing 0.1% Tween-20 (Sigma). The IP pellets were analysed by SDS-PAGE and Western blotted with corresponding antibodies to NHE3 and IKEPP.

Cell Lines

PS120 fibroblasts lack all endogenous plasma membrane NHEs, NHERF1 (minimal expression), NHERF2, PDZK1 and IKEPP. These cells, when stably expressing rabbit NHE3 with a C-terminal VSV-G protein epitope tag, are called PS120/NHE3 cells, as described (stable cell lines made using pcDNA 3.1; G418; Invitrogen) [23]. All PS120 lines 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, 400 µg/ml G418, and 10% fetal bovine serum in a 5% CO_2, 95% O_2 incubator at 37°C. PS120/NHE3 cells stably expressing IKEPP were also generated (using pcDNA 3.1; hygromycin) and cultured in the above medium supplemented with 600 µg/ml hygromycin.

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-acetoxymethyl ester (BCECF-AM, 5 μM; Molecular Probes, Eugene, OR), as described previously [24]. Cells were exposed to 40mM NH_4Cl alone or with 4-bromo-A23187 (0.5μM) during a 15-min dye loading, as described previously [24]. Cells were perfused initially with TMA^+ solution (130 mM tetramethylammonium chloride, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 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 pH_i recovery. At the end of each experiment, the fluorescence ratio was calibrated to pH_i using the high potassium/nigericin method [3, 24]. Na^+/H^+ exchange activity data was calculated as the product of Na^+-dependent changes in pH_i times the buffering capacity at each pH_i and individual points shown in figures are rates of Na^+/H^+ exchange calculated at multiple pH_i 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 V_{max} and $K'(H^+)_i$ in individual experiments. Means ± S.E. were determined from at least three separate experiments [24].

Measurement of Surface NHE3

To measure surface NHE3, PS120 cells were treated with either 4-bromo-A23187 or vehicle at 37°C and then surface-labeled with biotin at 4°C as described previously [25]. After rinsing in phosphate-buffered saline (150 mM NaCl and 20 mM Na_2HPO_4, pH 7.4), cells were incubated with NHS-SS-biotin (0.5 mg/ml; Pierce) in borate buffer (154 mM NaCl, 10 mM boric acid, 7.2 mM KCl, and 1.8 mM $CaCl_2$, pH 9) and then exposed to the quenching buffer (20 mM Tris-HCl and 120 mM NaCl, pH 7.4). Cells were lysed in 1 ml of N^+ buffer (60 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM KCl, 5 mM Na_3EDTA, 3 mM EGTA, and 1% Triton X-100), and the lysates were centrifuged at 13,000 × *g* for 30 min to remove insoluble cellular debris. Protein content in the supernatant was quantified by the BCA method, and equal amounts of cell lysate were incubated with streptavidin-agarose (Pierce) at 4°C. The remaining supernatant was retained as the intracellular fraction. The streptavidin-agarose beads were washed repeatedly in N^+ buffer, and the biotinylated proteins were solubilized in Laemmli's buffer. The total, intracellular, and surface fractions were resolved by SDS-PAGE and transferred to NC membrane, and NHE3 was quantified by labeling with anti-VSV-G antibody. The efficiency of NHE cell surface biotinylation is estimated to be at least 89% [26].

Sucrose density gradient centrifugation

PS120 cells were grown to confluency in 10-cm petri dishes. Cells were then solubilized in 1 ml of N^+ buffer, sonicated, and spun briefly to remove unbroken cells, nuclei, and cell debris. Solubilized cell extracts (4°C in 1% Triton X-100) 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* in a SW40i rotor, the gradients were fractionated (0.6 ml) from the bottom with a perfusion pump and NHE3 and IKEPP expression visualized by Western blot with anti-VSVG monoclonal antibody and anti-IKEPP monoclonal antibody, as described above. Individual bands from each fraction were quantified (integrated intensity) using the Odyssey Infrared Imaging System (Li-Cor) and percent total integrated intensity was calculated for each fraction.

Acceptor Photobleaching Förster Resonance Energy Transfer (FRET)

Fluorescence microscopy was performed as described previously on cells fixed in 3% paraformaldehyde [27, 28], with minor revisions. Cells were imaged on a Zeiss LSM 410 confocal microscope (Carl Zeiss, Thornwood, NY) using a 1.3 NA 100x Plan-neofluor objective. Cy3 and Cy5 fluorescence was excited using lasers at 534 and 633 nm and detected using appropriate filter sets (Cy3 filter cube: excitation 515-560 nm, 565 nm long pass dichroic, emission 573-648 nm; Cy5 filter cube: excitation 590-650 nm, 660 nm long pass dichroic, emission 663-738 nm) (Chroma Technology, Brattleboro, VT). No fluorescence was observed from a Cy3-labeled specimen using the Cy5 filters, nor was Cy5 fluorescence detected using the Cy3 filter set.

In the present study, the acceptor bleaching FRET method was used, as described in detail [27, 28]. Briefly, the energy transfer was detected as an increase in donor (Cy3) fluorescence (dequenching) after complete photobleaching of the acceptor (Cy5) fluorophore. The validity of using the acceptor photobleaching technique to quantify FRET depends on the fact that the only factor that can lead to a difference in donor fluorescence in the presence and absence of acceptor is energy transfer. Cells labeled only with Cy5-IKEPP were used to determine the minimum time required to completely bleach the Cy5. Typically, Cy5 was completely photobleached in 5 minutes of continuous laser excitation (633 nm) at full power using a Cy5 filter set. Under these conditions, ~99% of the Cy5 was bleached from the entire field of view, and no Cy3 bleaching or energy transfer occurred in control samples labeled with Cy3-(VSV)-G only. To perform an imaging FRET experiment, cells were stained with both Cy3-(VSV)-G and Cy5-IKEPP. The image of Cy3-NHE3 (donor) in the presence of Cy5-IKEPP (acceptor) was obtained using a Cy3 filter set. Next, the image of Cy5-IKEPP was obtained using a Cy5 filter set. Then the same fluorescent field of view was continuously illuminated for 5 min (at full laser power) at the Cy5 excitation wavelength and the image of Cy5 fluorescence after photobleaching was taken. Next another image of donor Cy3-(VSV)-G fluorescence was obtained using a Cy3 filter set. Images were collected from 10 different places from a single monolayer, stored on computer disk and fluorescence intensity of donor before and after acceptor photobleaching was measured for 50 identical 10x10 pixel regions of interest in each individual image using Metamorph (Universal Imaging Corp.) with macros. FRET efficiency was calculated as:

$E = (I_{D2} - I_{D1}) / I_{D2}$

where I_{D1} and I_{D2} are the donor fluorescence intensities before and after acceptor photobleaching, respectively [27]. FRET efficiency E was analyzed and plotted as a function of acceptor

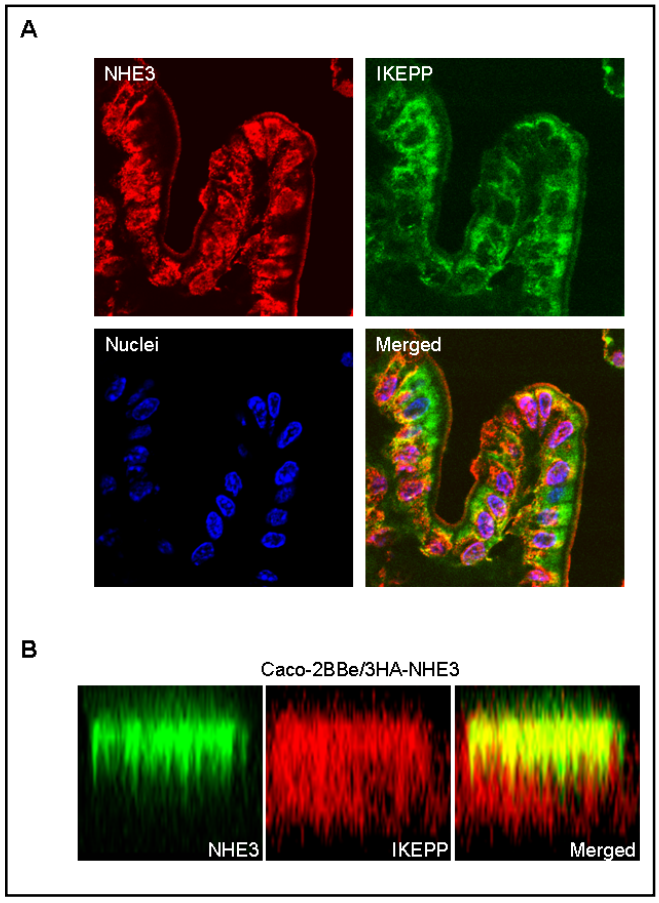


Fig. 1. NHE3 and IKEPP co-localize in intestinal epithelial cells. A. In paraffin-embedded mouse ileal sections, NHE3 and IKEPP co-localized in a juxtanuclear compartment and in the BB as determined by immunofluorscence confocal microscopy. B. NHE3 and IKEPP also demonstrated co-localization in the polarized intestinal epithelial cell model, Caco-2BBe. In Caco-2BBe cells infected with adenovirus 3HA-tagged NHE3 construct, NHE3 was localized predominantly to the BB. IKEPP appeared to co-localize with NHE3 but also exhibited localization in a sub-apical compartment which is consistent with results previously demonstrated by Scott et al in Caco-2 cells [13].

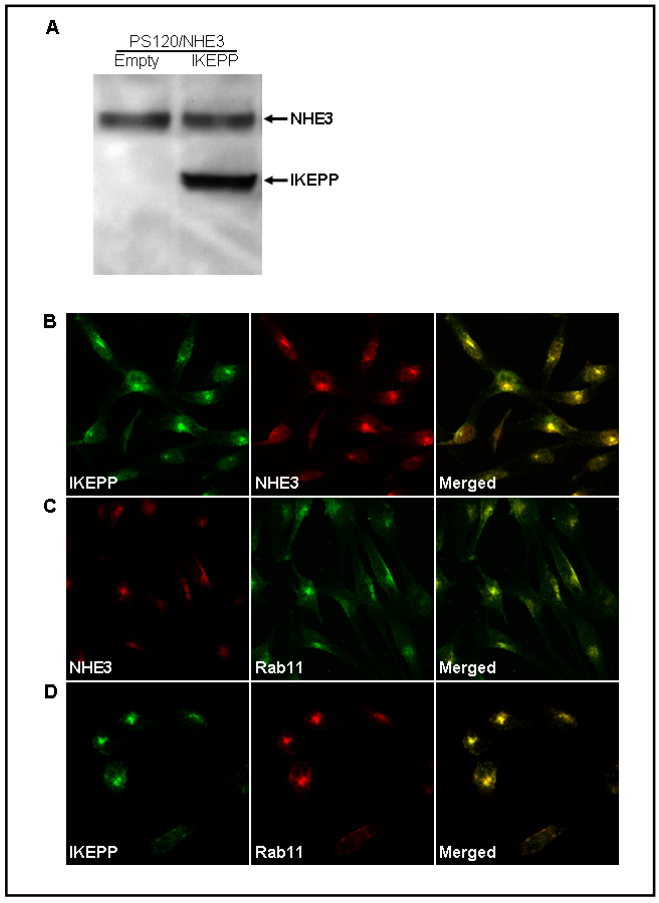


Fig. 2. IKEPP is predominantly localized intracellularly in PS120 cells. A. Expression of NHE3 and IKEPP in PS120 cells stably expressing NHE3 and IKEPP. PS120/NHE3/empty cells do not express IKEPP. B. In PS120 cells co-expressing NHE3 and IKEPP, co-localization occurs intracellularly. C. NHE3 is primarily localized to the recycling endosome, as indicated by the intracellular marker, Rab11. D. Similarly, IKEPP also co-localizes with intracellular Rab11.

fluorescence intensity.

Statistics

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

Results

Localization of NHE3 and IKEPP in intestinal epithelial cells

By light microscopy, IKEPP is expressed in the brush border and a cytoplasmic region adjacent to the BB of mouse intestinal epithelial cells [13]. We performed immunofluorescence studies in the mouse ileum to determine whether NHE3 and IKEPP co-localize in intestinal epithelial cells (Fig. 1). In paraffin-embedded mouse ileal sections, NHE3 was expressed in the BB as well as in juxtanuclear compartments. IKEPP co-localized with NHE3 mostly in juxtanuclear compartments and also in the BB of mouse intestinal epithelial cells (Fig. 1A). Similar studies were performed in the polarized intestinal epithelial cell model, Caco-2BBe (Fig. 1B). In Caco-2BBe cells expressing a 3HA-tagged NHE3 construct, endogenous IKEPP protein co-localized with NHE3 at

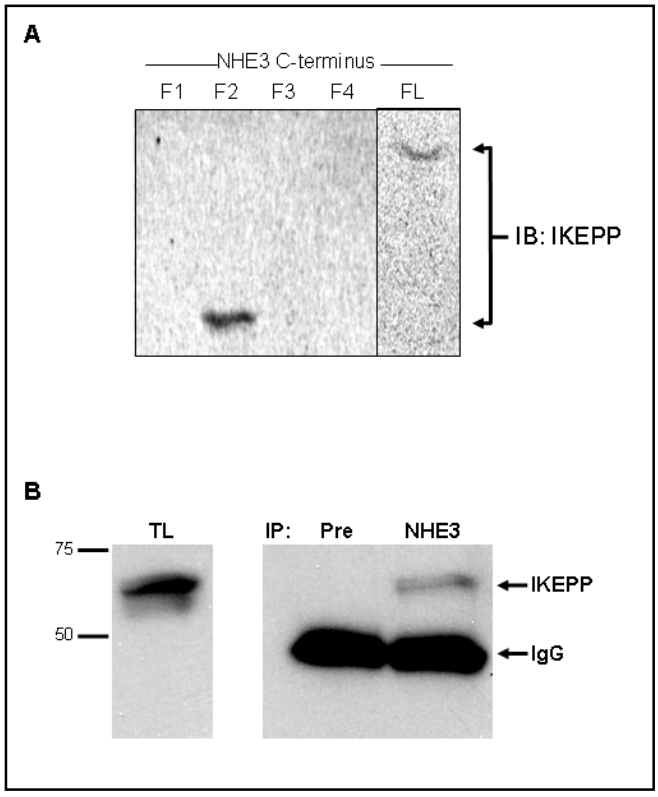


Fig. 3. IKEPP binds NHE3 C-terminus between aa 590-667 and associates in vivo. A. Purified protein fragments of the C-terminus of NHE3 were generated, separated on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with purified recombinant human IKEEP protein and visualized by autoradiography. Protein overlay assays demonstrate that purified recombinant human IKEPP binds to the full-length C-terminal fragment of NHE3. Futhermore, IKEPP interacts only with the F2 region of NHE3 (aa 590-667). B. Immunprecipitation of NHE3 from PS120/NHE3/IKEPP cells precipitated IKEPP while immunoprecipitation with preimmune serum did not.

the apical pole (Fig. 1B). NHE3 and IKEPP localization appeared to be predominantly BB; however, IKEPP also exhibited sub-apical localization, which is consistent with previous findings demonstrated by Scott et al [13].

Expression and Localization of IKEPP in PS120/NHE3/IKEPP cells

In order to test whether IKEPP plays a role in NHE3 regulation, we used the NHE3 and NHERF family deficient PS120 fibroblast model to stably express NHE3 and IKEPP. As a control, we also generated PS120 cells stably expressing NHE3 and pcDNA3.1 empty vector. As shown in Fig. 2A, western blot demonstrated that PS120/NHE3/empty and PS120/NHE3/IKEPP cells express similar levels of NHE3. In order to determine the cellular localization of IKEPP, we performed immunofluorescence in PS120/NHE3/IKEPP cells. In PS120/NHE3/IKEPP cells, IKEPP and NHE3 co-localize intracellularly (Fig. 2B) in a Rab11-positive juxtanuclear compartment (Fig. 2C, D). IKEPP was not found to co-localize with a marker of the ER, calnexin (data not shown). While NHE3 and IKEPP appear to colocalize to the same intracellular compartment, we next asked whether NHE3 and IKEPP directly bind.

IKEPP directly binds NHE3 between aa 589-667

To assess direct physical interaction of NHE3 and IKEPP, protein overlay assays were employed using the full-length C-terminus of NHE3 (aa. 475-832) as well as the C-terminus divided into four contiguous fragments. IKEPP bound to NHE3 directly. This interaction occurred in the full-length C-terminus of NHE3 as well as in the F2 C-terminal fragment (aa. 590-667) of NHE3 (Fig. 3A). The F2 fragment is the same region previously shown to bind NHERF1 and NHERF2 [22]. Thus IKEPP can directly bind NHE3 *in vitro*.

In addition to in vitro binding, we also performed co-immunoprecipitation studies in PS120/NHE3/IKEPP cells to determine if NHE3 and IKEPP associate in vivo. In this experiment, VSVG-tagged NHE3 was immunoprecipitated from PS120 cell lysates and expression of IKEPP was determined by western blot. The results of this study showed that immunoprecipitated NHE3 co-precipitated IKEPP whereas the preimmune control did not (Fig. 3B). Based on these data, NHE3 and IKEPP directly bind and associate in vivo. Therefore, the next study determined whether IKEPP had a role in regulation of NHE3 activity.

Elevation of $[Ca^{2+}]_i$ stimulates NHE3 activity in PS120 cells co-expressing IKEPP

Na^+/H^+ exchange was measured in PS120 cells using the pH-sensitive dye, BCECF. In PS120 cells only expressing NHE3, elevation of $[Ca^{2+}]_i$ using 4-bromo-A23187 had no effect on NHE3 activity (Fig. 4A). However, in PS120 cells stably expressing NHE3 and IKEPP, A23187 treatment significantly increased NHE3 activity, by more than 40% (Fig. 4B, C) over the same time course (15min).

To examine mechanisms of stimulated NHE3 activity, we determined whether the increase in NHE3 activity was associated with changes in plasma membrane expression of NHE3. Under basal conditions, NHE3

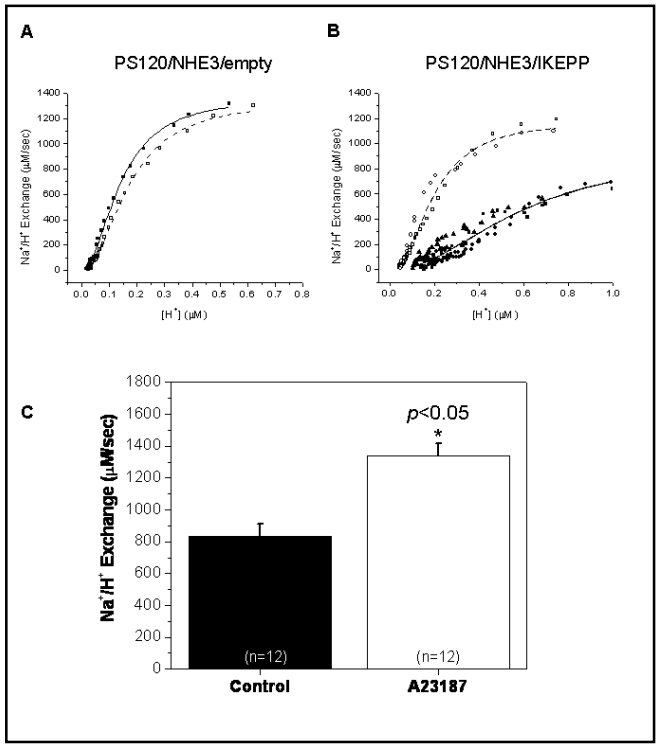


Fig. 4. Elevated $[Ca^{2+}]_i$ stimulates NHE3 activity in PS120 cells expressing IKEPP. A. Na^+/H^+ exchange was measured in PS120/NHE3 expressing IKEPP or empty vector control, using the pH-sensitive dye, BCECF, which was loaded into cells during 15 minute incubation with 40mM NH_4Cl. In PS120 cells only expressing NHE3, elevation of $[Ca^{2+}]_i$ by the calcium ionophore, 4-bromo-A23187, had no effect on NHE3 activity. A23187 treatment in PS120 cells co-expressing NHE3 and IKEPP, resulted in increased NHE3 activity. Panels A and B represent measurements taken from single experiments, closed circles = vehicle, open circles = A23187. C. Bar graph summarizes the mean and SE of 12 experiments for each condition. P value is in comparison with control (paired t test).

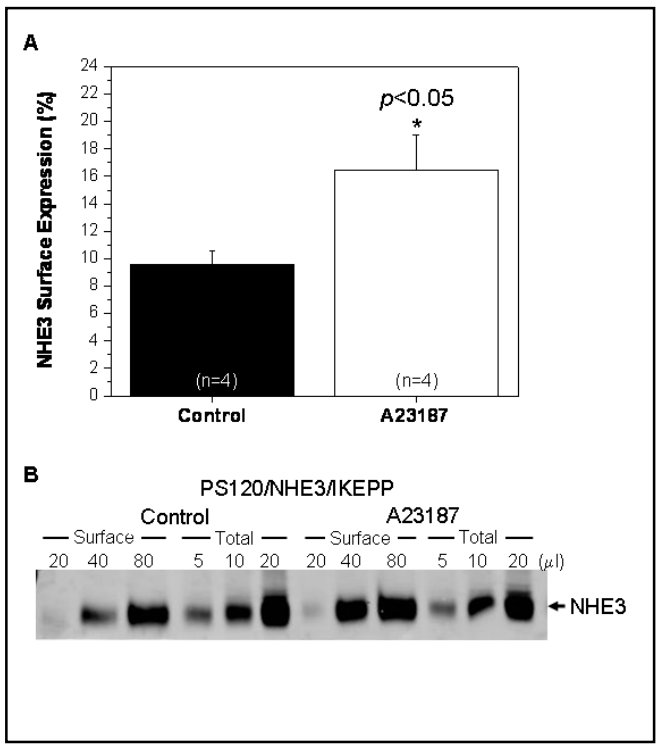
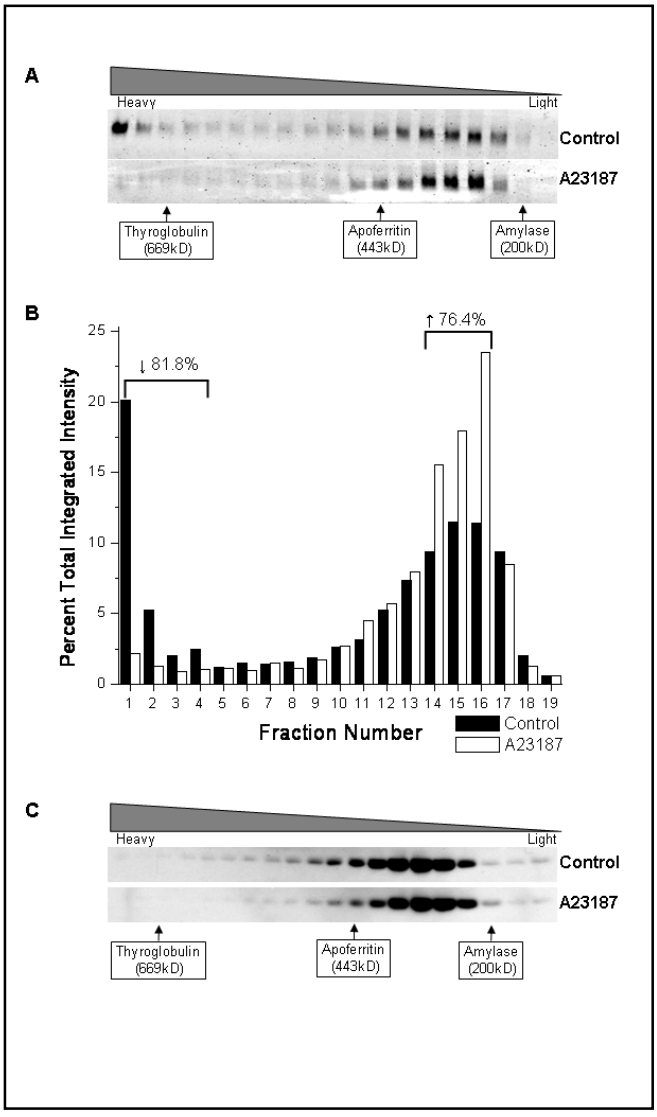


Fig. 5. Stimulation of NHE3 activity is due to increased surface expression of NHE3. PS120 cells expressing NHE3 and IKEPP were grown to 70-80% confluency in 10cm Petri dishes and treated with vehicle or A23187 for 15 minutes. Cells were washed and incubated twice with Sulfo-NHS-SS biotin (50mM) for 20 minutes. Cell lysates were collected and subjected to avidin precipitation and analyzed by western blot analysis. A. Surface biotinylation of PS120/NHE3V/IKEPP cells showed that basal levels of surface NHE3 (9.6 ± 1.1%) increased by 63.5 ± 19.2% ($p<0.05$) in response to elevated calcium (16.5 ± 2.6%). Results are from four independent experiments. B. Results from a single experiment.

surface expression was 9.6% ± 1.1 and increased by ~64% ($p<0.05$) to 16.5% ± 2.6 in cells treated with A23187 (Fig. 5A, B).

Inhibition of NHE3 activity by carbachol was previously shown to be associated with an increase in the size of NHE3-containing multi-protein complexes [29]. In contrast, sucrose density gradients of PS120/NHE3/IKEPP cells exposed initially to 4-bromo-A23187 (0.5 μM) for 20 min demonstrated a decrease in the size of NHE3 containing complexes compared to control (Fig. 6A). Under basal conditions, NHE3 containing complexes primarily exist in two distinct groups of fractions: fraction numbers 1-4 (800-1000kDa) and 14-17 (200-400kDa) (Fig. 6B). After treatment with A23187, there was an 81.8% decrease in the amount of NHE3 containing complexes in fractions 1-4 (Fig. 6B). Conversely, there was a 76.4% increase of NHE3 containing complexes in fractions 14-16 (Fig. 6B). Similarly, IKEPP distributed in multiple protein complexes under basal conditions, and IKEPP containing protein complexes decreased in size in PS120/NHE3/IKEPP cells treated with A23187 when compared to control (Fig. 6C). Thus both NHE3 and IKEPP are in dynamic complexes which are altered by elevated $[Ca^{2+}]_i$.

Fig. 6. IKEPP mediated stimulation of NHE3 activity under elevated calcium conditions is associated with decreased size of NHE3 and IKEPP containing complexes. A. Cells were seeded onto 10cm Petri dishes and treated as described above. Whole cell lysate from PS120/NHE3/IKEPP cells were collected after 15 minutes of treatment with either vehicle or 0.5 μM A23187. Cells lysates were centrifuged through a sucrose density gradient as described by Li et al. [29] with proteins of known size separated on an identical parallel gradient as standards. NHE3 containing complexes were identified by western blot. NHE3 was shifted from heaviest sized complexes in fractions (1-4) to lighter fractions (14-16), as quantified using an Odyssey fluorescent imager. B. Percent total integrated intensity in fractions 1-4 decreased by nearly 82% in PS120/NHE3/IKEPP cells treated with A23187. In contrast, fractions 14-16 exhibited a greater than 76% increase in NHE3 complexes when compared to control. C. Similarly, IKEPP containing complexes moved from heavier to lighter fractions in PS120 cells expressing NHE3 and IKEPP after treatment with A23187.



IKEPP remains localized to the Rab11-positive recycling endosome under elevated calcium conditions

In PS120/NHE3/IKEPP cells treated with A23187, NHE3 activity and surface expression were significantly increased. These data suggest that under elevated calcium conditions, increased NHE3 activity is due to increased exocytosis. In order to determine whether IKEPP also traffics to the plasma membrane after A23187 treatment, we performed immunofluorescence studies. As shown in Fig. 2D and Fig. 7A, IKEPP localizes to the Rab11-positive recycling endosome under basal conditions. Similarly, after elevation of $[Ca^{2+}]_i$ by A23187, IKEPP and Rab11 remained colocalized (Fig. 7B). The results of this study suggests that IKEPP binds NHE3 under basal conditions and this interaction is decreased after elevation of $[Ca^{2+}]_i$ to allow NHE3 trafficking to the plasma membrane. Moreover, there is no evidence that IKEPP moves from the recycling (Rab11-positive) compartment under elevated $[Ca^{2+}]_i$ conditions.

IKEPP and NHE3 interactions decrease in response to elevated calcium

Cy3/Cy5 FRET was used to confirm direct protein-protein interactions of NHE3 and IKEPP in PS120 cells under basal conditions and to test whether that interaction was dynamic with elevated $[Ca^{2+}]_i$. In this approach NHE3 (Cy3) and IKEPP (Cy5) were shown to directly bind one another in an intracellular compartment in PS120 cells under basal conditions.

Acceptor photobleaching FRET was used to demonstrate direct NHE3/IKEPP association and to assess any changes that occur in NHE3/IKEPP interactions with elevated $[Ca^{2+}]_i$. Using confocal microscopy, initial images of Cy3-labeled NHE3 (Fig. 8Aa', e') and Cy5-labeled IKEPP (Fig. 8Ac', g') were obtained focusing on intracellular sites of localization. NHE3 fluorescence (Fig. 8Ab', f') was measured once Cy5 fluorescence (Fig. 8Ad', h') was no longer detectable. Quantification of fluorescence energy transfer was performed under basal and elevated $[Ca^{2+}]_i$ examining intracellular sites of NHE3 and IKEPP localization by using 0.5 μm xy sections, which were at least 0.5 μm from all plasma membranes. Analysis of intracellular regions revealed that NHE3 and IKEPP exhibit FRET under basal conditions and with elevated $[Ca^{2+}]_i$. However, the total percent energy transfer between IKEPP and

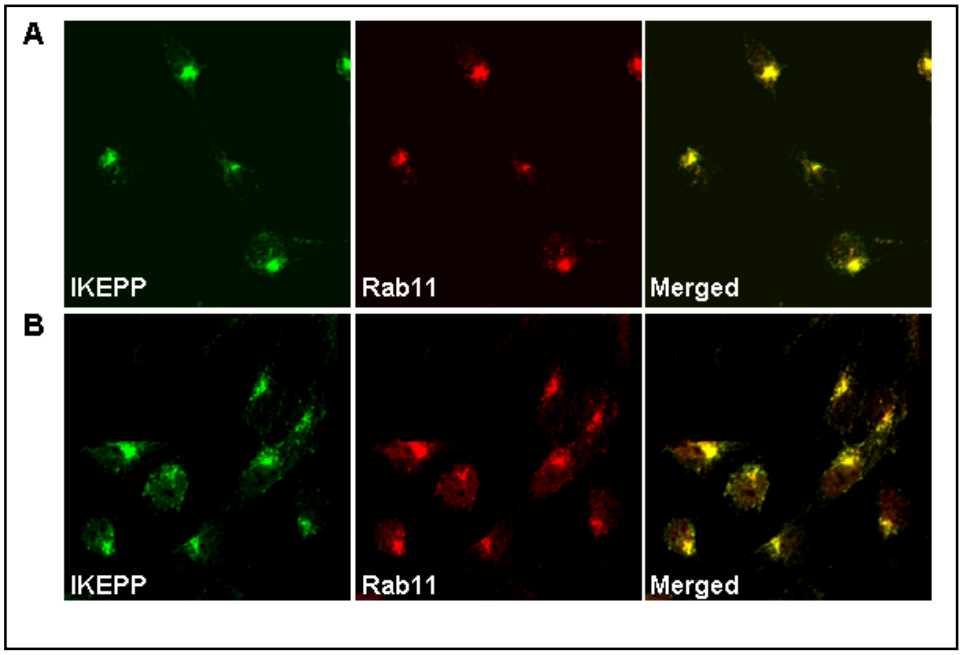


Fig. 7. IKEPP remains localized to the Rab11-positive recycling endosome under elevated calcium conditions. A. IKEPP colocalizes with Rab11 in PS120/NHE3/IKEPP cells as determined by immunofluorescence confocal microscopy. B. Treatment of PS120/NHE3/IKEPP with 0.5μM A23187 did not alter IKEPP localization with Rab11.

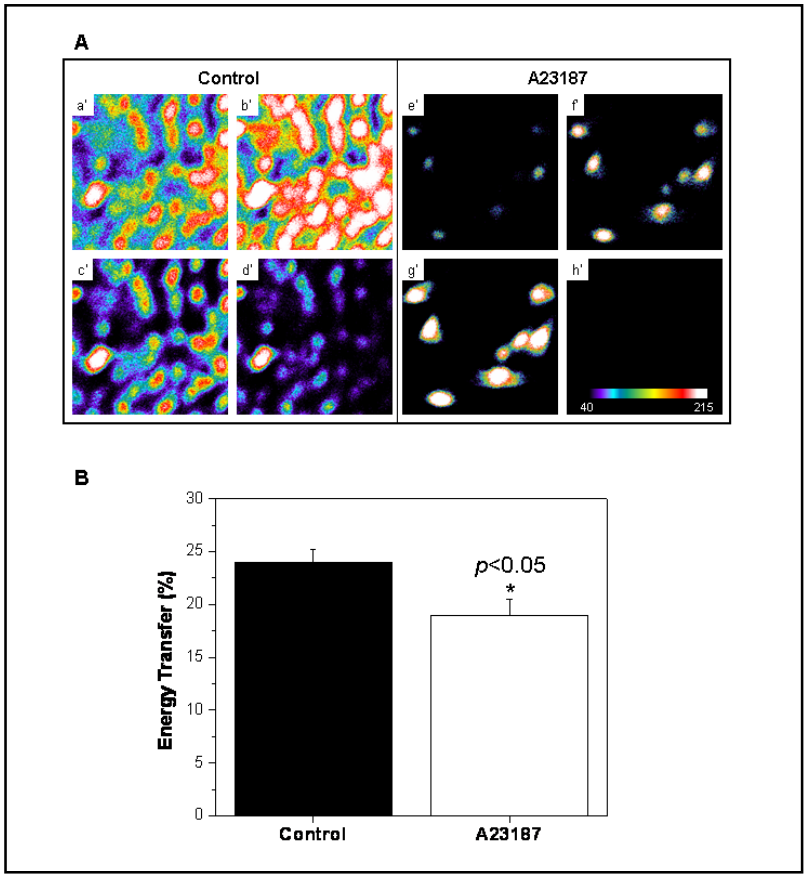


Fig. 8. NHE3 and IKEPP directly associate intracellularly as determined by Förster Resonance Energy Transfer and this association decreases in response to elevated calcium. A. PS120 cells expressing NHE3 and IKEPP were immunostained with antibodies conjugated to Cy3 (NHE3) or Cy5 (IKEPP) and treated with vehicle or A23187. Using confocal microscopy, initial fluorescent images were captured before photobleaching (Fig. 8Aa',c',e',g'). Cy-5 labeled IKEPP signal was bleached with continuous laser excitation until Cy 5 fluorescence was nearly abolished (Fig. 8Ad', h'). Images were captured after Cy5 photobleaching (Fig. 8Ab', f') and analyzed for changes in fluorescent intensity using the MetaMorph image analysis software. B. The total percent energy transfer was significantly (p<0.05) decreased in PS120/NHE3/IKEPP cells treated for 10 minutes with A23187.

NHE3 decreased in response to elevated $[Ca^{2+}]_i$ (Fig. 8B). These data suggest that NHE3 and IKEPP physically interact intracellularly under basal $[Ca^{2+}]_i$ conditions and there is a decreased association in the NHE3 and IKEPP association after $[Ca^{2+}]_i$ was elevated for 15 min.

Discussion

The results of the current study (1) provide evidence for a role of IKEPP in calcium regulation of NHE3 activity and (2) establish direct intracellular protein-protein

interactions between NHE3 and IKEPP. The current study demonstrated that IKEPP directly binds the C-terminus of NHE3 *in vitro* (overlay assay) and *in vivo* (FRET). Confocal microscopy studies identified that this intracellular compartment was probably the recycling endosome, as determined by Rab11 co-localization and previous studies demonstrating NHE3 localized to the recycling compartment and the plasma membrane (in PS120 cells) and the BB (Caco-2BBe cells and mouse ileum). Moreover, the association of NHE3 and IKEPP is dynamic based upon FRET experiments demonstrating a decreased association of intracellular NHE3 and IKEPP under elevated $[Ca^{2+}]_i$ conditions. In PS120 cells co-expressing NHE3 and IKEPP, elevation of $[Ca^{2+}]_i$ by the ionophore, A23187, caused a significant increase in NHE3 activity. This effect was not observed in PS120 cells expressing NHE3 alone. Increased Na^+/H^+ exchange was due to increased plasma membrane expression of NHE3. The current study also showed that under basal conditions NHE3 and IKEPP are in some similar sized large multiprotein complexes as determined by sucrose density gradient centrifugation. However, when $[Ca^{2+}]_i$ was elevated, some of the NHE3-containing complexes were reduced in size and, based upon FRET studies, no longer bound to IKEPP. These findings support previous studies demonstrating that specific NHERF family PDZ proteins are necessary for calcium mediated regulation of NHE3 activity [5, 18], but demonstrate that the nature of the effect on NHE3 depends on which NHERF family protein is involved.

Of the four NHERF family members, the role of NHERF1 and NHERF2 in NHE3 regulation has begun to be studied and recently a role for NHERF3 in NHE3 regulation has been described in the mouse intestine [30, 31]. NHERF3 appears to be necessary to set basal NHE3 activity in the mouse colon [31]. However, the role of IKEPP is not well described. Studies of NHERF1, NHERF2, and NHERF3 in regulation of NHE3 activity have previously identified roles in $[Ca^{2+}]_i$ regulation of NHE3 [3, 5, 18, 25, 30]. Although the NHERF family members share homology within their PDZ domains, each member exhibits distinct effects on NHE3 activity under elevated calcium conditions (NHERF1, no effect; NHERF2 and NHERF3, NHE3 inhibition; IKEPP, NHE3 stimulation). These differences may be due to specificity of protein-protein interactions of the various NHERF proteins and differences in proteins they bring intoNHE3 related signaling complexes. Importantly, the use of a calcium ionophore in these studies allowed concentration on effects downstream of the elevation of $[Ca^{2+}]_i$ rather than on the receptors which are linked to $[Ca^{2+}]_i$ elevation and the immediately associated signaling molecules.

Previous studies in our laboratories have demonstrated that elevation of $[Ca^{2+}]_i$ inhibits NHE3 by an α-actinin-4 and PKCα dependent process, which requires a complex involving NHERF2 [5]. In addition, NHERF3 also reconstitutes calcium-mediated inhibition of NHE3 activity by a mechanism different than NHERF2 [Zachos et al., unpublished data]. Furthermore, other studies in the rabbit ileum have demonstrated that elevation of $[Ca^{2+}]_i$ through activation of the muscarinic receptor (M3R) inhibits electroneutral NaCl absorption by a PKC dependent mechanism, with NHE3 being the major transporter involved [17]. While the results of the current study seem to contradict previous findings of the role of calcium in inhibition of NHE3 activity, Lee-Kwon et al. [18] demonstrated previously that lysophosphatidic acid (LPA) stimulates NHE3 in OK cells by a NHERF2 dependent mechanism which was associated with elevated $[Ca^{2+}]_i$ and was calcium dependent (prevented by BAPTAM). The NHERF2 requiring step identified was activation of $PLC\beta_3$ (bound to NHERF2) by the LPA receptor with a higher and more prolonged elevated Ca^{2+} response occurring in cells expressing NHERF2 [32]. Thus, the results of these studies provide a precedent for involvement of a NHERF protein in calcium-mediated stimulation of NHE3 activity. Similar to NHERF2, IKEPP exists in multi-protein containing complexes. However, future studies are needed to determine whether other proteins are involved in the IKEPP mediated stimulation of NHE3 activity.

The results of the current study demonstrate a role for IKEPP in Ca^{2+} regulation of the surface expression of NHE3. Furthermore, quantitative data compiled from acceptor photobleaching experiments conclude that IKEPP does not traffic with NHE3 from the endosome to the plasma membrane as part of this regulation. This result is supported by recent studies demonstrating trafficking of CFTR and MRP2 to the apical membrane utilizing mutagenesis experiments which disrupted PDZ binding sites [33-35]. The clearest mechanistic understanding of the IKEPP effect comes from the FRET experiments which demonstrated that elevated Ca^{2+} caused a decreased association of intracellular NHE3 and IKEPP. Elevation of $[Ca^{2+}]_i$ has also been demonstrated to release NHERF3 from NHE3 in PS120 cells which results in decreased NHE3 activity and surface expression [Zachos et al., unpublished data]. Previous studies have suggested that NHERF1 anchors transport proteins (e.g. CTFR, NaPiIIa) to the apical

membrane of epithelial cells. In fact, preliminary studies in Caco-2BBe cells in which NHERF3 knockdown by shRNA significantly reduces BB NHE3 expression suggests that NHERF3 may be necessary for anchoring NHE3 to the apical membrane [Zachos et al., unpublished data]. Based upon results from the current study, we conclude that IKEPP anchors NHE3 in the recycling compartment and releases NHE3 with elevated $[Ca^{2+}]_i$ to allow NHE3 to traffic to the plasma membrane. Whether IKEPP plays a similar role in intestinal epithelial cells remains to be determined.

The role of calcium in signal transduction and protein trafficking remains incompletely understood. Calcium regulates various steps in the endocytic removal of integral membrane proteins as well as specific fusion events that take place during exocytic insertion of intracellular cargo to the plasma membrane. For example, elevation of intracellular calcium through activation of the insulin receptor results in increased surface expression of the GLUT4 transporter [36, 37]. A role for calcium has been conclusively demonstrated at the late stages of exocytosis, for instance involving the synaptotagmin family [38, 39]. Interestingly, the results of the current study also suggest a role for calcium regulation at the level of the Rab11-positive recycling endosome in NHE3 regulation as implied by the FRET experiments, although additional roles in delivery of NHE3 to the plasma membrane are likely. Future studies in mammalian epithelial tissues will be necessary to determine the role of the NHERF proteins in the effect of elevated $[Ca^{2+}]_i$ on endocytosis and exocytosis.

The PS120 cell model does not express the four members of the NHERF family as occurs in epithelial Na^+-absorptive cells. However, the goal of the current study was to determine whether IKEPP alone could reconstitute calcium regulation of NHE3 activity. Future studies in epithelial cells will be needed to understand the role of IKEPP in calcium regulation of NHE3 in the presence of the full family of NHERF proteins which are present in these cells.

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