Tissue-specific Regulation of Sodium/Proton Exchanger Isoform 3 Activity in Na\(^+/\)H\(^+\) Exchanger Regulatory Factor 1 (NHERF1) Null Mice

CAMP INHIBITION IS DIFFERENTIALLY DEPENDENT ON NHERF1 AND EXCHANGE PROTEIN DIRECTLY ACTIVATED BY CAMP IN ILEUM VERSUS PROXIMAL TUBULE\(^*\)

Received for publication, March 5, 2007, and in revised form, May 31, 2007 Published, JBC Papers in Press, June 19, 2007, DOI 10.1074/jbc.M701910200

Rakhilya Murtazina, Olga Kovbasnjuk, Nicholas C. Zachos, Xuhang Li, Yueping Chen, Ann Hubbard, Boris M. Hogema, Deborah Steplock, Ursula Seidler, Kazi M. Hoque, Chung Ming Tse, Hugo R. De Jonge, Edward J. Weinman, and M. Donowitz

From the Departments of Medicine, Physiology, and Cell Biology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; Department of Biochemistry, Erasmus University Medical Center, 3000 CA Rotterdam, The Netherlands; Department of Medicine, University of Maryland School of Medicine and Medical Staff, Department of Veteran Affairs, Baltimore, Maryland 21201, and Department of Medicine, Hannover University School of Medicine, 2 D-30419 Hannover, Germany

The multi-PDZ domain containing protein Na\(^+/\)H\(^+\) Exchanger Regulatory Factor 1 (NHERF1) binds to Na\(^+/\)H\(^+\) exchanger 3 (NHE3) and is associated with the brush border (BB) membrane of murine kidney and small intestine. Although studies in BB isolated from kidney cortex of wild type and NHERF1 \(^{-/-}\) mice have shown that NHERF1 is necessary for cAMP inhibition of NHE3 activity, a role of NHERF1 in NHE3 regulation in small intestine and in intact kidney has not been established. Here a method using multi-photon microscopy with the pH-sensitive dye SNARF-4F (carboxysemitrifluoromethyltrifluoromethane-4F) to measure BB NHE3 activity in intact tissue is described. cAMP inhibition of NHE3 activity in ileum and wild type and NHERF1 \(^{-/-}\) ileum and wild type kidney cortex were inhibited by cAMP, whereas the cAMP effect was abolished in kidney cortex of NHERF1 \(^{-/-}\) mice. cAMP inhibition of NHE3 activity in these two tissues is mediated by different mechanisms. In ileum, a protein kinase A (PKA)-dependent mechanism accounts for all cAMP inhibition of NHE3 activity since the PKA antagonist H-89 abolished the inhibitory effect of cAMP. In kidney, both PKA-dependent and non-PKA-dependent mechanisms were involved, with the latter reproduced by the effect on an EPAC (exchange protein directly activated by cAMP) agonist (8-(4-chlorophenylthio)-2'-O-Me-cAMP). In contrast, the EPAC agonist had no effect in proximal tubule in NHERF1 \(^{-/-}\) mice. These data suggest that in proximal tubule, NHERF1 is required for all cAMP inhibition of NHE3, which occurs through both EPAC-dependent and PKA-dependent mechanisms; in contrast, cAMP inhibits ileal NHE3 only by a PKA-dependent pathway, which is independent of NHERF1 and EPAC.

The Na\(^+/\)H\(^+\) exchanger regulatory factor (NHERF) family of multi-PDZ domain proteins consists of four homologous and evolutionarily related proteins (1–4). In renal proximal tubule and small intestinal epithelial cells, all four NHERF proteins are present in the apical domain either in the brush border (BB) or just below it (Refs. 5 and 6; this study). Although named after their role in regulation of NHE3, these proteins affect many classes of proteins. Among them, transport proteins are the most frequently identified, and the most intensely studied function of the NHERF proteins is in regulation of these transport proteins (7). The mechanisms of actions involved are multiple in the regulation of transport proteins. These include directing delivery of proteins to the plasma membrane from the synthetic pathway and/or recycling system, affecting plasma membrane retention, and more directly affecting turnover number (7–12). In addition, NHERF family members bring a wide variety of signaling molecules into complexes, some of which only bind to one or another of the NHERF family, whereas others bind to all.

Because of the presence of multiple (if not all) NHERF family members in the proximal tubule and small intestinal apical membrane domains, it has proven difficult to attribute the NHERF regulatory functions to specific members of this gene family in vivo. In contrast, simple cells lacking most or all NHERF family members have been useful in determining the functions of individual NHERF proteins (13–18). This has been done in most detail related to regulation of NHE3 activity. For instance, cAMP inhibition of NHE3 mimics some postprandial aspects of digestion (induced by secretin) and can be reproduced in PS120 fibroblasts by expression of either NHERF1 or NHERF2; whereas in the absence of both proteins, cAMP has a...
EPAC Effect on Renal NHE3 Is Dependent on NHERF1

reduced or no effect on NHE3 activity (13, 19). The situation is more complicated in epithelial cell models, such as Caco-2 and OK cells, which are used as intestinal and renal proximal tubule Na⁺ absorptive cell models, respectively. Both cells express multiple NHERF family members in the apical domain (5, 20). Knockdown studies of each NHERF family member have the potential to determine the role of each individual family member, although few studies have been reported using this approach (21–23). Nonetheless, these models do not reproduce the complexity of intact tissue, in which NHERF family proteins may be present in adjacent cells of different types that interact. Knocking out individual and multiple NHERF family members in mouse intestine and proximal tubule is a promising approach, and NHERF1 and NHERF3/PDZK1 knock-out mice have been partially characterized (1, 24–27).

Renal proximal tubule and small intestine both use BB NHE3 to absorb the majority of luminal Na⁺. It has been assumed that regulation of NHE3 by common agonists acts by similar mechanisms in these two epithelia. However, suggestions have also been made that regulation of NHE3 by trafficking is very different between these two tissues, with the possibility that NHE3 trafficking only occurs between the microvilli and intervillus clefts in proximal tubule, whereas NHE3 traffics in the intestine by the more conventional plasma membrane→ intervillus clefts (clathrin-coated pits and lipid rafts)→ common and recycling endosomes→ return to surface and some entry to degradative pathways of late endosomes→ microvesicular bodies→ lysosomes (28, 29). Comparisons of regulation of NHE3 between small intestine and proximal tubule by the same agonist has the potential to provide further insights into how NHE3 is regulated differently in these two epithelia.

A role for NHERF1 in cAMP inhibition in the mouse proximal tubule has been suggested based on comparison of wild type and NHERF1−/− mouse NHE3 activity in BB membrane vesicle preparations and in primary cultures of these cells (26, 30). However, studies of the effect of cAMP on NHE3 activity in intact renal proximal tubule or ileal Na⁺ absorptive cells from wild type compared with NHERF−/− mice have not been reported. This study describes development of a method using multi-photon microscopy to quantitate apical membrane NHE3 activity in intact mouse small intestine and proximal tubule and demonstrates important differences between cAMP inhibition of NHE3 in these two epithelial tissues and in the dependence of NHE3 regulation on NHERF1.

EXPERIMENTAL PROCEDURES

Materials—SNARF-4F acetoxymethyl ester was from Invitrogen; HOE694 was from Sonafi-Aventis; 8-pCPT-2′-O-Me-cAMP was from BioLog; salts and other chemicals were from Sigma or Fisher at the highest grade available. Rabbit polyclonal antibody to NHE3 (Ab1381 and Univ. of Arizona), NHERF1 (Ab5199), and NHERF2 (Ab2570) were previously characterized (31, 32, 13). Anti-ezrin and anti-actin antibodies were from Sigma; anti-EPAC1 antibodies were from Santa Cruz.

Animals—Male NHERF1−/− mice bred into a C57Bl/6 background (Charles River) for at least six generations were produced from heterozygotes as initially reported (26). NHERF1−/− mice and wild type male C57Bl/6 mice were studied between 10 and 14 weeks of age. The mice were maintained under standard light and climate conditions in the animal facility of the Johns Hopkins University School of Medicine with ad libitum access to water and chow. Experiments with animals were carried out using protocols approved by the Animal Use Committee of the Johns Hopkins University.

Isolation of jejunum, ileum, and kidney cortex for Na⁺/H⁺ Exchange Activity Assays—Mice were Briefly Anesthetized with ether and then Sacrificed by cervical dislocation. The abdomen was immediately opened by midline incision and proximal jejunum (~2 cm in length starting ~1 cm distal to ligament of Treitz), and distal ileum (~3 cm in length ending 1 cm proximal to the ileo-cecal junction) were excised and placed immediately in cold “Na⁺ buffer” (138 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 25 mM glucose, and 20 mM HEPES, pH 7.4) and opened along the antimesenteric border. 6–8-mm pieces were mounted with Krazy Glue (Elmer’s Products Inc., Columbus, OH) onto a glass coverslip with the mucosal surface facing up. The kidney was cut in half with a razor blade, the capsule was removed, and the cortex was excised using a razor blade. 1–1.5-mm-thick slices were glued onto coverslips. All preparations were performed on ice. The glue used for mounting had no autofluorescent signal and did not affect viability of cells since the percent of cells taking up propidium iodide at the end of the experiment was similar to non-glued tissue maintained in Na⁺ buffer (data not shown).

CAMP Treatment before Study for Electron Microscopy or Immunochemistry—Ileum or kidney segments were preincubated for 10 min at 37 °C in Ringer-HCO₃, 10 mM glucose, gassed at 95% O₂, 5% CO₂, and then exposed at 37 °C to 100 μM 8-Br-cAMP or an equal volume of H₂O (as vehicle control) for 30 min and then examined for electron microscopy or immunohistochemistry.

Measurement of Mouse Jejunum, Ileal, and Renal Cortical NHE3 Activity by Multi-photon Microscopy—The wavelength for excitation of SNARF-4F for conventional confocal microscopy is between 488 and 530 nm with emission 580 and 640 nm. The emission wavelengths are the same for the two-photon microscope, whereas the wavelength for dye excitation was determined empirically. Wavelengths between 740 and 800 nm produced the largest emission fluorescence without visible tissue damage with average power from the Ti:Sa laser ~800–860 milliwatts and pulse bandwidth ~16–18 nm, corresponding to <50 fs pulse duration at 80 MHz repetition rate. This excitation was attenuated by the optical system and by a combination of neutral density filters such that the average intensity at the focal plane was ~10 milliwatts.

SNARF-4F Loading and Imaging—The protocol for imaging intracellular pH of intact mouse jejunum, ileum, or kidney cortex using multi-photon microscopy is described under “Results.” By using a 60×/1.00 water immersion objective (Nikon), the images of the jejunal and ileal villus or renal cortical proximal tubule cells loaded with the “dual emission” pH-sensitive dye, SNARF-4F in Na⁺ buffer, pH 7.4, were visualized using a multi-photon laser scanning microscope (MRC-1024MP, Bio-Rad) powered by a wide band, infrared (780 nm) combined photo-diode pump laser and mode-locked titanium-
sapphire laser (Tsunami Ti:Sa laser, Spectra-Physics, Mountain View, CA). The 8-bit images were recorded and stored, after which fluorescence intensity was calculated off-line using MetaMorph 5.0 r1 software (Molecular Devices Corp.) as described below.

Jejunum, ileum, and renal cortical slices were loaded with 20 μM SNARF-4F in Na⁺ buffer at 37 °C for 35 min with 95% O₂, 5% CO₂ gassing. The coverslip with the glued tissue was then placed in a perfusion chamber (RC-21BDW, Warner Instrument), mounted onto a heated microscope stage at 25 °C (PH series, Warner Instrument), and perfused using a peristaltic pump (Imatec; Reglo, Switzerland) at 1 ml/min with Na⁺ buffer for 15 min at room temperature. Tissue was then acidified using 100 mM HOE694 to eliminate the contributions of NHE1 and NHE2. As described under Results, reagents of interest (100 μM 8-Br-cAMP, 100 μM 8-pCPT-2’-O-Me-cAMP, 50 or 1 μM H-89) were added to all perfusion buffers. 1 mM probenecid was in all perfusates to prevent SNARF-4F leakage (33–35). The leakage of dye was greater in small intestine than in kidney. In all tissues the leakage of dye was increased when specimens were perfused with Na⁺-free buffer (NMDA buffer). This suggested that extracellular Na⁺ retained the SNARF-4F inside the cells. Because SNARF-4F leakage in kidney was much less than in small intestine, one slice of kidney cortex was used for both control and treated conditions sequentially (time control studied in parallel), whereas in experiments in small intestine, two separate pieces of tissues obtained from two animals were used for the control/treated conditions.

**SNARF-4F Emission**—For images for each optical section (small intestine and kidney cortex), 0–50 μm from villus tip and cut surface, both at 10 μm steps (Fig. 1, A and B), were taken at 580 and 640 nm and stored. These conditions allowed quantifiable signals to be studied at depths up to 40–50 μm from the villus tip or cortical surface. Below that, the signal became too dim to obtain quantitative ratiometric data.

**Analysis of Collected Images**—Optical images for analysis were taken typically starting at 20 and 30 μm from the tip of villus or 10 μm below the cut surface of the kidney cortex to avoid villus cells potentially close to shedding into the small intestinal lumen and damaged cortical cells from the slice preparation. Regions of interest (ROI) including regions for measurement of background were randomly chosen in 2–3 individual villi (Fig. 1C) or proximal tubules. Fluorescence intensity in gray levels that correspond to relative amounts of SNARF-4F for each ROI (16–21 ROIs per time point) for both 640- and 580-nm emissions was calculated using MetaMorph. The intensity of background was subtracted from each chosen ROI. The 640/580 ratio for each ROI was calculated, average value of ROIs were determined for each time point, and 640/580 ratios over time were determined followed by conversion to pH values with internal pH standards using Microsoft Excel. The Na⁺/H⁺ exchange activity of NHE3 was determined as the initial rate in pH change by calculating the initial steep pH slope after the addition of Na⁺ buffer using linear curve fit analysis (Origin 6.0) and presented as ΔpH/min.

**Immunofluorescence Staining of Ileum for NHE3, NHERF1, and NHERF2**—Tissue samples exposed to cAMP or control solutions were obtained as described above and fixed in 3.5% paraformaldehyde in PBS at 4 °C and paraffin-embedded. Histological sections (4 μm thick) were mounted onto Superfrost microscope slides (Fisher) and heat-fixed. Slides were microwaved for antigen recovery in 10 mM sodium citrate buffer, pH 6 (Sigma) 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 in PBS for 30 min at room temperature. Sections were incubated for 1 h at room temperature and then for 48 h (4 °C) with polyclonal NHERF1, NHERF2, or NHE3 antibodies, diluted 1:500 in 5% normal goat serum-PBS. In parallel studies using the same animals, kidney

**FIGURE 1.** A representative example of multi-photon microscope optical sections of tissues loaded with 20 μM SNARF-4F for 35 min at 37 °C. The images were taken at pHout = 7.3 and t = 25 °C. The dye loaded in tissue was excited at 780 nm with emission ratio imaging at 580 and 640 nm using a 60 ×/1.00 objective lens (Nikon). Panel A represents optical sections of ileum, ROI (black ellipses) including a region corresponding to background (white ellipses) were chosen. Fluorescence intensity in gray levels that corresponds to the SNARF-4F signal in each ROI for both wavelengths was measured using MetaMorph 5.0 software. The intensity ratios 640/580 of each region minus background were calculated, and the mean ± S.E. was determined for each time point.
cortex slices also were immediately fixed in fresh PLP (2% paraformaldehyde, 0.875 M l-lysine-HCl, 0.1 M sodium-meta-periodate) for 2 h at room temperature. Tissues were rinsed twice in 0.5 M sucrose, 0.1 M sodium phosphate for 10 min before leaving tissues in rinsing solution overnight at 4 °C. Cortical kidney slices were placed in plastic embedding molds and filled with OCT embedding medium (Tissue-Tek). Molds were then immediately submerged in isopentane and placed on dry ice. After excess solvent had evaporated, molds were stored at −80 °C until sectioned. 8-μm kidney sections were fixed in ice-cold 95% methanol for 10 min. Sections were rinsed three times in 1 X PBS and blocked in 5% normal goat serum in PBS containing 0.2% Triton X-100 and 0.02% sodium azide for 30 min at room temperature. Kidney sections were incubated with a polyclonal primary antibody to NHE3 (Ab1381) diluted 1:100 in blocking solution for 1 h at room temperature. With both methods of fixation, ileal and kidney sections were then washed twice in PBS for 10 min and incubated with anti-rabbit Alexa-fluor secondary antibodies, each diluted 1:1000 for 1 h at room temperature. Methods of fixation, ileal and kidney sections were imaged using a Zeiss LSM510 confocal microscope (63342, Invitrogen), and mounted with Gel Mount (Sigma). Ileal villus cells in control solutions in vitro were obtained as described above and fixed by immersion in ice-cold 1.6–2% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.4, for 60 min. During that time tissue was cut into smaller segments (~3 mm on a side). After rinsing in 0.1 M sodium cacodylate, 3.5% sucrose, the tissue was incubated in reduced osmium (1.5% potassium ferrocyanide, 1% OsO4 in the same buffer) for 60 min on ice, rinsed several times in water, dehydrated through a series of graded EtOH, and embedded in EPON 812. Ultrathin sections were prepared, stained with uranyl acetate and then lead citrate, and viewed with a Hitachi 7600 microscope. Microrvillar lengths were determined at a magnification of ×25000 at the microscope using the “measurement” tool. Three independent experiments were performed, and the microvilli of 5–13 cells were measured for each treatment.

Electron Microscopy of Ileum from Wild Type and NHERF1−/− Mice—Tissue samples exposed to cAMP or control solutions in vitro were obtained as described above and fixed by immersion in ice-cold 1.6–2% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.4, for 60 min. During that time tissue was cut into smaller segments (~3 mm on a side). After rinsing in 0.1 M sodium cacodylate, 3.5% sucrose, the tissue was incubated in reduced osmium (1.5% potassium ferrocyanide, 1% OsO4 in the same buffer) for 60 min on ice, rinsed several times in water, dehydrated through a series of graded EtOH, and embedded in EPON 812. Ultrathin sections were prepared, stained with uranyl acetate and then lead citrate, and viewed with a Hitachi 7600 microscope. Microrvillar lengths were determined at a magnification of ×25000 at the microscope using the “measurement” tool. Three independent experiments were performed, and the microvilli of 5–13 cells were measured for each treatment.

Ileal and Proximal Tubule Total Membrane Preparation/Immunoblot from Wild Type and NHERF1−/− Mice—Ileum (distal half of small intestine) and kidney cortex were excised from animals. Ileum were rinsed with ice-cold 0.9% saline and opened along the anti-mesenteric borders. Kidney cortices were rinsed in 0.9% saline and then transferred into homogenization buffer A (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM Na3VO4, 50 mM NaF). Ileal villus cells were scraped with a glass slide and placed into homogenization buffer B (60 mM mannitol, 2.4 mM Tris, pH 7.1, 1 mM EGTA, 2 mM Na3VO4, 1 mM β-glycerol phosphate, 1 mM phenylalanine). Protease inhibitor mixture (Sigma) 1:100 was added to buffers A and B, and phosphoramidon (1:1000) was added only to buffer B. Scraped ileal villus cells in buffer B and kidney cortex in buffer A were homogenized at 4 °C with a Polytron (10 times for 10 s at speed 5 with a 20 s interval between each burst) followed by homogenization of samples in a glass-Teflon homogenizer. The homogenates were centrifuged at 4000 rpm for 10 min at 4 °C to remove cell debris and nuclei. Supernatants were then centrifuged at 40000 rpm for 60 min, and total membrane pellets were collected. The resulting total membranes were resuspended in buffer A for kidney preparations and in buffer B for ileal mucosa. The protein concentrations in total membrane were measured with BCA (Sigma). Samples were analyzed with SDS-PAGE Western blotting using primary antibodies for EPAC1, ezrin, actin, or NHERF1 with fluorescently labeled secondary goat anti-mouse IRDye TM800 antibodies (Rockland). The fluorescence intensity of detected protein bands was quantified by the Odyssey system (LI-COR).

Statistics—Values are presented as the mean ± S.E. Statistical significance was determined using Student’s unpaired and paired t-tests. p values <0.05 were considered significant.

RESULTS

Calibration of the Fluorescence Response of SNARF-4F in Kidney Cortex and Small Intestine at Different pH Values—Multiphoton technology rather than conventional confocal microscopy was selected to measure intracellular pH because it caused minimal bleaching in the bulk of the sample and induced less phototoxicity, which made longer study periods possible. These studies typically lasted on single pieces of tissue for a total of 2.5 h in jejunum and ileum and 3.5 h in the kidney cortex.

The pKa value for small intestine and kidney cortex for SNARF-4F acetoxymethyl ester was experimentally determined. Because the fluorescence response of SNARF-4F in solution and intracellularly is often different (pKa value for free SNARF-4F is 6.4 (Invitrogen “Molecular Probes” manual), the SNARF-4F response in kidney cortex and in ileum was calculated by the K+ ionophore/nigericin (10 μM) method (36) in the presence of 150 mM K+ extracellularly to equilibrate the intracellular pH with the pH-controlled extracellular media. Fig. 2 shows the calibration of the pH response as the ratio of fluorescence intensities of SNARF-4F measured at two different emission wavelengths 640 and 580 nm for kidney cortex (panels A) and ileum (panels C). The calculation of the fluorescence response of the dye to different pH values for kidney cortex (panel B) and ileum (panel D) yields apparent pKa values ~6.8 and ~7.4, respectively.

Basal NHE3 Activity—The experimental protocol is shown in Fig. 3, A and D. Typically, before acidification, fluorescence was analyzed at 3 time points 1 min apart, and similar numbers of time points and timing were analyzed after prepulse with NH4Cl and during incubation in NMDA buffer to determine basal Na+/H+ exchange activity under acidified conditions. Then 12–15 readings were obtained at 1-min intervals during pH recovery in Na+ buffer. Calibration of 640/580 ratio was performed using the K+/nigericin method for external pH using pH standards of 6.1–6.3, 6.7–6.8, and 7.3–7.4 (36). NHE3 activity was defined as the initial rate of ΔpH/Atime, which was 5-(N-ethyl-N-isopropyl) amiloride-sensitive (100 μM) during the Na+-dependent alkalinization when Na+ (138 mM) solution perfused the ileal luminal or kidney cortex surface after establishing a baseline pH in the absence of Na+. NHE1 and
NHE2 were inhibited by inclusion of 50 μM HOE694 in both Na⁺-free and Na⁺ perfusates. Initial rates of NHE3 activity were highest in renal proximal tubule > jejunum > ileum. Mean ΔpH/min ± S.E. for kidney proximal tubule was 0.47 ± 0.10 (n = 4), for jejunum was 0.25 ± 0.10 (n = 4), and for ileum was 0.14 ± 0.02 (n = 20). Thus, multi-photon microscopy/SNARF-4F allows intact tissue quantitation of NHE3 activity in murine small intestine and renal proximal tubule with renal NHE3 activity >> small intestine (jejunum > ileum). Fig. 4C compares the amount of NHE3 in mouse BB from proximal tubule and jejunum BB, with again much more in proximal tubule than jejunum (see “Discussion” for comparison of renal cortex and jejunal NHE3 amount and activity).

cAMP Inhibits NHE3 Activity in Kidney Proximal Tubules and Ileum, but the cAMP Is Differently Dependent on NHERF1—It has been shown that NHERF1 or NHERF2 are necessary to enable cAMP inhibition of NHE3 activity in PS120 cells (13, 37), whereas NHERF1 is necessary for cAMP inhibition of NHE3 in mouse proximal tubule based on studies of isolated BB membrane vesicles and primary cultures of proximal tubule cells (30). No evaluation has been carried out in small intestine of cAMP inhibition of NHE3 activity in NHERF1 null mice nor are studies reported in intact kidney cortex. The effect of 8-Br-cAMP (100 μM) added to the luminal surface of ileum and perfused over the cell surface of kidney cortex on NHE3 activity was determined using multi-photon microscopy (Fig. 3, B, C, and E; F shows data of a representative experiment, and Fig. 4 shows the means ± S.E. of more than four animals). As shown in Fig. 4A, basal NHE3 activity was very similar in kidney cortex from WT and NHERF1−/− mice. It was previously shown that proximal tubule NHE3 activity and the amount of protein were similar in wild type and NHERF1−/− mice (30). 8-Br-cAMP inhibited NHE3 activity by 45 ± 12% in proximal tubule of wild type mice but failed to affect NHE3 activity in intact renal cortex of the NHERF1 null mice.

The level of basal NHE3 activity in ileum from WT and NHERF1−/− mice was similar and lower than in kidney proximal tubules (Fig. 4, B compared with A). Note similar ileal NHE3 amounts in wild type and NHERF1−/− mice (Fig. 4C). In both wild type and NHERF1−/− ileum, cAMP caused similar inhibition of NHE3, WT (55 ± 7%), and NHERF1 null mice (64 ± 5% ns) (Fig. 4B).

Electron Microscopy of Ileal BB—Because of the report of abnormal morphology and composition of intestinal BB in another NHERF1 null mouse model in C57Bl/6 background (38) and its potential consequences for transport, electron microscopy of mouse ileum of WT and NHERF1−/− mice were compared on tissue obtained under basal and 8-Br-cAMP-treated conditions. Supplemental Fig. S1 shows that the ileal morphology from NHERF1−/− mice appeared similar to wild type. The ultrastructure of the ileal brush border and lengths of microvilli were indistinguishable. Shown in supplemental Fig. S1 are results from 12-week adult mice, but similar normal brush borders were present in ileum of 4–5-week-old wild type and NHERF1−/− mice (data not shown). No structural changes at the ileal BB of WT or NHERF1−/− mice were observed after 30 min of exposure to 100 μM 8-Br-cAMP (data not shown).

Immunofluorescence Imaging of Ileum (Supplemental Fig. S2) and Proximal Tubule (Supplemental Fig. S3)—To determine whether 8-Br-cAMP treatment altered NHE3, NHERF1, or NHERF2 localization in ileum and proximal tubules of wild type or NHERF1−/− mice, immunofluorescence/confocal microscopy studies were performed. Intact ileum and kidney cortical slices were taken from wild type or NHERF1−/− mice and treated in vitro with vehicle or 100 μM 8-Br-cAMP for 30 min, formalin fixed, and paraffin-embedded. In parallel studies
kidney sections were also frozen before processing. In the wild type ileum, NHE3, NHERF1 and NHERF2 were all predominantly expressed in the BB of villus epithelial cells (supplemental Fig. S2, upper panels). NHE3 and NHERF1 appeared to have a uniform pattern of distribution within the BB, whereas NHERF2 was more punctate. Endothelial cell distribution of NHERF2, as previously described (5), is indicated by arrows. In the ileum of NHERF1−/− mice (supplemental Fig. S2, upper panels, NHERF1 knock-out (NHERF1 KO), NHERF1 expression completely disappeared from the BB, thus confirming specificity of the antibody. There did appear to be residual cytoplasmic expression in the NHERF1−/− ileum, which we attribute to nonspecific staining. Also, NHERF2 staining was decreased in the NHERF1−/− ileum but still expressed in the same location in the BB. Although this reduction may be related to some cross-reactivity in the detection of NHERF1 and NHERF2 with the antibody used,3 we conclude that NHERF2 is present in ileal BB. Also, the NHE3 localization is somewhat changed in the NHERF1−/− ileum compared with that in wild type, being more intracellular or subapical (supplemental Fig. S2, upper panel, NHERF1 knock-out (NHERF1 KO, right)). Treatment of ileum from WT or NHERF1−/− mice with 100 μM 8-Br-cAMP caused more intracellular NHE3 in both wild type and NHERF1−/− mice but had no significant effect on localization of NHERF1 or NHERF2 (supplemental Fig. S2, lower panels).

In the proximal tubule of wild type mice, NHERF1 and NHERF2 localization were not affected by the method of fixation; however, NHE3 localization was clearer with frozen tissue processing, and results from this processing are shown (supplemental Fig. S3). NHE3, NHERF1, and NHERF2 were localized to the BB of renal proximal tubule epithelial cells. NHE3 and NHERF2 expression and localization were similar in wild type and NHERF1−/− proximal tubule in basal conditions (supplemental Fig. S3, upper panel). After treatment with 100 μM 8-Br-cAMP, NHE3 localization in the BB of the proximal tubule from wild type mice was somewhat more diffuse but was not decreased compared with untreated tissue, whereas localization of NHERF1 and NHERF2 did not change (supplemental Fig. S3, lower panels). No changes in NHE3 localization in BB after cAMP treatment were observed in the NHERF1−/− proximal tubule.

To confirm the specificity of the NHE3 antibodies, intact ileum (supplemental Fig. S4) was isolated from NHE3−/− mice, fixed, paraffin-embedded, and immunostained. As shown in supplemental Fig. S4, NHE3 staining is absent in the BB of ileum compared with localization in supplemental Fig. S2, 3 B. Hogema, H. de Jonge, and M. Donowitz, unpublished information.
cAMP Acts Only via PKA in Mouse Ileum but by Both PKA and EPAC in Mouse Proximal Tubule—Further studies were undertaken to determine the basis for the differences between ileum and proximal tubule in cAMP inhibition of NHE3 in mouse Na\(^+\) absorptive epithelia. cAMP can act by both PKA and the more recently recognized PKA-independent EPAC pathways. Given the recent evidence that in mouse proximal tubule, cAMP inhibition of NHE3 is partially EPAC-dependent, which does not involve phosphorylation of NHE3 (39), we asked whether inhibition of PKA prevented the cAMP-mediated inhibition of NHE3 in either ileum or proximal tubule. The PKA antagonist H-89 was initially used. Pretreatment of ileum and NHERF1\(^{-/-}\) mice with H-89 (50 \(\mu\)M), present in the dye loading buffer with 10 \(\mu\)M added to all perfused solutions (2.5 h to onset of perfusion), did not significantly alter basal ileal NHE3 activity in wild type mice or in NHERF1\(^{-/-}\) mice but totally prevented 8-Br-cAMP inhibition of ileal NHE3 activity in both wild type and NHERF1\(^{-/-}\) mice (Fig. 5, A and B). Because 50 \(\mu\)M H-89 may inhibit protein kinase C (PKC) in addition to PKA (40, 41), we repeated these studies using 1 \(\mu\)M H-89, a concentration that inhibits PKA but not PKC. Even at 1 \(\mu\)M, H-89 did not alter basal ileal NHE3 activity but totally prevented cAMP inhibition of NHE3 (data not shown).

Different results from the ileal studies were seen when we examined the H-89 effect on renal proximal tubule NHE3 activity. As shown in Fig. 5C H-89 did not alter basal proximal tubule NHE3 activity in WT mice. 8-Br-cAMP still inhibited NHE3 in the presence of 50 \(\mu\)M H-89. The effect, however, was reduced by \(\sim\)50%. This result suggests a difference of cAMP effect on ileal and proximal tubule NHE3 and shows that a non-PKA component contributes to the 8-Br-cAMP effect on renal proximal tubule but not ileal NHE3 activity.

cAMP Acts Partially by EPAC in Proximal Tubule but Not Ileum—Total blockade of 8-Br-cAMP inhibition of ileal NHE3 activity by H-89 suggested that EPAC did not contribute to cAMP inhibition of NHE3 activity in ileum. In contrast, that H-89 caused a \(\sim\)50% reduction of 8-Br-cAMP inhibition of NHE3 in kidney cortex suggested there might be a potential role for EPAC in NHE3 regulation. To establish a role for EPAC, an EPAC selective agonist 8-pCPT-2’-O-Me-cAMP with minimal effect on PKA (42, 43) was used. 8-pCPT-2’-O-Me-cAMP (200 \(\mu\)M) was added to the dye-loading buffer for 30 min at 37 °C and also was present in all perfused buffers (50 \(\mu\)M). As shown in Fig. 6A, the EPAC agonist inhibited wild type mouse proximal tubule NHE3 activity and the magnitude of the inhibitory effect was \(\sim\)50% that of the effect of 8-Br-cAMP (Fig. 6A compared with Figs. 4A and 5C). In contrast, the EPAC agonist failed to affect ileal NHE3 activity (Fig. 6C).

Immunoblots of mouse ileum and renal cortex total membrane preparations were performed to determine whether differences in EPAC1 expression explained the differential EPAC agonist effects on NHE3. As shown in supplemental Fig. S5, EPAC was expressed in both ileum and proximal tubule, where it appeared as a double band. EPAC appeared to be decreased in amount in total membrane in NHERF1\(^{-/-}\) ileum but not renal cortex.

**DISCUSSION**

In this study we report development of a method of measuring NHE3 activity in intact murine small intestine and proximal tubule that uses multi-photon microscopy and the intracellular pH dye SNARF-4F (see supplemental “Discussion”). Despite some limitations of the SNARF-4F dye, the two-photon microscopic approach allowed determination of initial rates of NHE3 activity in small intestine and kidney cortex. The major finding in this study is that cAMP regulates NHE3 in the murine ileum and proximal tubule by very different mechanisms...
EPAC Effect on Renal NHE3 Is Dependent on NHERF1

**A** ileum, Wild type

| 8-Br-cAMP | H-89 | + | + | - | + | - | + | + | + |
|----------|------|---|---|---|---|---|---|---|---|
| 8-Br-cAMP | H-89 | + | + | + | + | + | + | + | + |

**B** ileum, NHERF1 (-/-)

| 8-Br-cAMP | H-89 | + | + | - | + | - | + | + | + |
|----------|------|---|---|---|---|---|---|---|---|
| 8-Br-cAMP | H-89 | + | + | + | + | + | + | + | + |

**C** Kidney cortex, WT

| 8-Br-cAMP | H-89 | + | + | - | + | - | + | + | + |
|----------|------|---|---|---|---|---|---|---|---|
| 8-Br-cAMP | H-89 | + | + | + | + | + | + | + | + |

**FIGURE 5.** cAMP inhibition of NHE3 activity in ileum from WT (A) and NHERF1-/- mice (B) is entirely PKA-dependent, but in C proximal tubule is only partially PKA-dependent. Ileum was loaded with 20 μM SNARF-4F in Na+/containing buffer in the presence of vehicle or 8-Br-cAMP (200 μM), H-89 (50 μM in panels A and B), or both 8-Br-cAMP and H-89 for 35 min at 37 °C. 8-Br-cAMP (100 μM), H-89 (10 μM), or both reagents together were present in all perfusion buffers. Initial rates of NHE3 activity were determined. Data shown are the mean ± S.E. of four experiments for ileum from WT mice (panel A) and three experiments for NHERF1-/- mice (panel B). P values are a comparison of 8-Br-cAMP effect in the absence of H-89 versus presence and also effect of H-89 on basal NHE3 activity (unpaired t test). cAMP inhibition of NHE3 in ileum of wild type mice is similar in magnitude to the effect in NHERF1-/- ileum. H-89 totally prevented cAMP inhibition of ileal NHE3 activity, NS, not significant. C, kidney cortex was loaded with 20 μM SNARF-4F for 35 min at 37 °C in Na+/buffer also containing combinations of 100 μM 8-Br-cAMP, 50 μM H-89, or both reagents together in all perfusion buffers. NHE3 activity is represented as initial rate (μEqH/min). Bars and errors bars show the mean ± S.E. of six experiments. P values are a comparison of 8-Br-cAMP effect in the absence of H-89 versus presence (paired t test, p < 0.05) and of the effect of H-89 on basal NHE3 activity (paired t test, NS).
NHERF2 of EPAC from proximal tubule lysate failed to demonstrate a physical association (data not shown). In unpublished studies, Bos et al. (45) report that ezrin binds to and co-precipitates EPAC and is responsible for bringing EPAC to the apical membrane of polarized epithelial cells. Because ezrin is decreased in the apical membrane of proximal tubule cells from NHERF1 null mice (39), it is possible that NHERF1 is necessary to bring EPAC to the apical membrane of proximal tubule via acting as an anchor for ezrin. The currently accepted model for AMP inhibition of NHE3 in ileum is that NHERF1 or NHERF2 presents PKAII to phosphorylate NHE3 via an PKA-anchoring protein (46). The PKA-anchoring protein is thought to be ezrin, which simultaneously binds to the NHERFs and to PKAII (47). The model of associating proteins for the EPAC1 effect on proximal tubule NHE3 may be similar to this model for PKAII, with EPAC1 in the apical domain replacing PKAII (46). The mechanism involved is speculated to involve apical membrane NHE3 that is not phosphorylated by PKAII but does involve a small G protein, Rap, the downstream mediator of EPAC function. Whether Rap1 is differentially expressed in proximal tubule and ileum is not known.

In a slight modification of the NHERF/ezrin/PKAII model (45), we showed here that NHERF1 is not necessary for cAMP inhibition of ileal NHE3. The explanation is not understood, although it is possible that another NHERF family member, NHERF2, for instance, which also binds to ezrin, or NHERF3/PDZK1, which is not thought to bind ezrin, substitutes for NHERF1. We favor a model in which NHERF2 substitutes for NHERF1, since expression of NHERF3/PDZK1 in ileal and renal BB is decreased in NHERF1 null mice. The distinction between ileum and proximal tubule may also be in different subcellular locations of NHERF2, which is subapical in kidney cortex (thought to be in areas of clathrin coated pit formation (5)) but appears to be in both BB and subapical area in ileum and also has a more punctate distribution than NHERF1. Combinations of knockouts of multiple NHERF family members will have to be examined to understand the dependence of cAMP inhibition of NHE3 on the NHERF family. It also remains unexplored whether NHERF3/PDZK1 or NHERF4/IKEPP act as PKA-anchoring proteins to allow cAMP inhibition of ileal NHE3 activity.

In addition, we found by immunofluorescence that the pool of NHE3 in the BB decreased and/or that intracellular NHE3 increased after cAMP in ileum but did not clearly change in the proximal tubule of wild type mice. Although a model of regulation of NHE3 by trafficking via regulated endocytosis and exocytosis is supported by studies in small intestine (28), most studies in intact proximal tubule have been interpreted to suggest that NHE3 traffics only between the microvilli and intervillos clefts and is not endocytosed in a regulated fashion (29). Our results of a broader distribution of NHE3 in proximal tubule BB after cAMP are consistent with this model.

The comparison of NHE3 expression in BB of renal proximal tubule and jejunum revealed that there was far more NHE3 expressed in proximal tubule (the immunoblot in Fig. 4C showed a greater signal for renal NHE3, but there was four times more protein loaded for jejunum, and the BB enrichment was approximately five times greater in jejunum using enrichment of alkaline phosphatase in BB compared with initial lysate as the criteria). This suggests at least ~20× more NHE3 expression in proximal tubule. NHE3 activity, however, was only approximately two times greater in proximal tubule than jejunum. We interpret this to suggest that basal regulation of NHE3 is very different in proximal tubule and small intestine (greater activity/molecule for NHE3 in small intestine) just as the regu-

![Figure 6.](image-url)

**FIGURE 6.** EPAC is an intermediate in cAMP regulation of NHE3 activity in mouse proximal tubule Na⁺ absorptive cells and acts by a NHERF1-dependent mechanism (panels A and B), but EPAC is not involved in cAMP regulation of NHE3 in ileum (panel C). Kidney cortex from WT and NHERF1 null mice were loaded with 20 μM SNARF-4F for 35 min at 37 °C in Na⁺ buffer. 200 μM 8-pCPT-2′-O-Me-cAMP was added during dye loading and was included in NH₄Cl, NMDA, and Na⁺ buffers at 50 μM along with 30 μM HOE 694 (except for NH₄Cl buffer). NHE3 activity is represented as initial rate (ΔpH/min). Results are the mean ± S.E. of four experiments for WT (panel A) and experiments in NHERF1 null mice (panel B). p values are comparison of control and 8-pCPT-2′-O-Me-cAMP-treated kidney cortex (paired t test), NS, not significant. Panel C, 8-pCPT-2′-O-Me-cAMP fails to affect NHE3 in wild type mouse ileum. Studies of ileum (Fig. 6C) are as in proximal tubule. EPAC agonist failed to affect ileal NHE3 activity. Results are the mean ± S.E. of three experiments.
EPAC Effect on Renal NHE3 Is Dependent on NHERF1

Endocytotic mechanisms by which cAMP inhibits NHE3 are very different between proximal tubule and small intestine.

This study also helped further characterize several of the tools used. The use of histological sections of NHE3−/− mouse ileum and proximal tubule helped show that the staining in the BB by the anti-NHE3 antibody is specific. Also, NHERF2 was demonstrated to be present in ileal BB as well as in renal proximal tubule. Previously, based on immunoblotting, it was questioned whether mouse ileum expressed NHERF2, especially given that when concentrated by BB preparation, NHERF2 antibody seems to recognize some NHERF1. Please note that in our immunofluorescence studies, in NHERF1−/− mice, whereas there was a decrease in amount of BB NHERF2 signal, there was residual BB NHERF2, strongly indicating the presence of NHERF2 in the ileal BB (supplemental Fig. S2).

The structure of the ileal Na+ absorptive cell brush borders from NHERF1 null mice on a C57Bl/6 background, as described here, were normal by light microscopy and electron microscopy. The BB of renal proximal tubules of these mice were also normal, as we previously reported (6). This finding was supported by the normal basal NHE3 activity in ileum and proximal tubule and the normal magnitude of cAMP inhibited ileal NHE3 activity reported here. This is different from the report of another NHERF1 knock-out mouse bred into the same C57Bl/6 background as in this study in which ileal Na+ absorptive cell BB had abnormal microvilli and terminal webs (38). Both NHERF1−/− mice have been documented as lacking NHERF1 message and protein, with the major difference in the studies being that the mice studied here were older (~12 weeks) than those in the other study (5 weeks). However, we repeated the electron microscopy studies in 4–5-week-old wild type mice that also had normal ileal brush borders.

It is known that regulated BB endocytosis occurs to a much larger extent in kidney proximal tubule than in small intestine and that regulation of NHE3 by endocytosis may involve different compartments in proximal tubule and ileum (discussed earlier), and now we demonstrate that the signaling molecules engaged (at least the role of NHERF1 and EPAC1) in response to elevation of cAMP differ between ileum and proximal tubule even though NHE3 is inhibited in both. These results set the stage for detailed mechanistic studies to understand how endocytosis of NHE3 occurs in both tissues as well as provide the opportunity to understand in more detail the role of the NHERF family in NHE3 regulation.

Acknowledgments—We acknowledge Brian O’Rourke, Division of Cardiology, Johns Hopkins University School of Medicine, and Marshall Montrose, Physiology Department, University of Cincinnati, for assistance in establishing the technical aspects of the multi-photon microscopic measurement of NHE3 activity. We thank Dr. Juergen Paenter for providing HOE694 (Sanofi-Aventis). We thank Dr. Fayaz Ghishman, University of Arizona School of Medicine, for anti-NHE3 antibody. We thank Dr. Gary Shull, University of Cincinnati School of Medicine, for ileal tissue sections from NHE3 null mice.

REFERENCES

1. Weinman, E. J., Cunningham, R., Wade, J. B., and Shenolikar, S. (2005) J. Physiol. (Lond.) 567, 27–32
2. Donowitz, M., Cha, B., Zachos, N. C., Brett, C. L., Sharma, A., Tse, C. M., and Li, X. (2005) J. Physiol. (Lond.) 567, 3–11
3. Thelin, W. R., Hodson, C. A., and Milgram, S. L. (2005) J. Physiol. (Lond.) 567, 13–29
4. Hernandez, N., Gisler, S. M., Bribanic, S., Deliot, N., Capuano, P., Wagner, C. A., Moe, O. W., Biber, J., and Murer, H. (2005) J. Physiol. (Lond.) 567, 21–26
5. Wade, J. B., Welling, P. A., Donowitz, M., Shenolikar, S., and Weinman, E. J. (2001) Am. J. Physiol. 280, C192–C198
6. Wade, J. B., Liu, J., Coleman, R. A., Cunningham, R., Steplock, D. A., Lee-Kwon, W., Pallone, T. L., Shenolikar, S., and Weinman, E. J. (2003) Am. J. Physiol. 285, C1494–C1503
7. Shenolikar, S., Voltz, I. W., Cunningham, R., and Weinman, E. J. (2004) Physiology (Bethesda) 19, 362–369
8. Cao, T. T., Deacon, H. W., Reczek, D., Bretheser, A., and von Zastrow, M. (1999) Nature 401, 286–290
9. Gage, R. M., Kim, K. A., Cao, T. T., and von Zastrow, M. (2001) J. Biol. Chem. 276, 13712–13720
10. Gage, R. M., Matveeva, E. A., Whiteheart, S. W., and von Zastrow, M. (2005) J. Biol. Chem. 280, 3305–3313
11. Puthenveedu, M. A., and von Zastrow, M. (2006) Cell 127, 113–124
12. Guggino, W. B., and Stanton, B. A. (2006) Nat. Rev. Mol. Cell Biol. 7, 426–436
13. Yun, C. H., Oh, S., Zizak, M., Steplock, D., Tsaos, S., Tse, C. M., Weinman, E. J., and Donowitz, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3010–3015
14. Kim, J. H., Lee-Kwon, W., Park, J. B., Ryu, S. H., Yun, C. H., and Donowitz, M. (2002) J. Biol. Chem. 277, 23714–23724
15. Mahon, M. J., Donowitz, M., Yun, C. C., and Segre, G. V. (2002) Nature 417, 858–861
16. Lee-Kwon, W., Kwano, K., Choi, J. W., Kim, J. H., and Donowitz, M. (2003) J. Biol. Chem. 278, 16494–16501
17. Lee-Kwon, W., Kim, J. H., Choi, J. W., Kwano, K., Cha, B., Dartt, D. A., Zoukhir, D., and Donowitz, M. (2003) Am. J. Physiol. 285, C1527–C1536
18. Cha, B., Kim, J. H., Hut, H., Hogema, B. M., Nadarajah, J., Zizak, M., Cavel, M., Lee-Kwon, W., Lohmann, S. M., Smolenski, A., Tse, C. M., Yun, C., de Jonge, H. R., and Donowitz, M. (2005) J. Biol. Chem. 280, 16642–16650
19. Emami, S., Chastre, E., Bodere, H., Gespach, C., Bataille, D., and Rosselin, G. (1996) Peptides (Elmsford) 7, Suppl. 1, 121–127
20. Zachos, N. C., Li Hodson, C., Chen, Y., Milgram, S., and Donowitz, M. (2005) Gastroenterology 131, 177
21. Takahashi, Y., Morales, F. C., Kreimann, E. L., and Georgescu, M. M. (2006) EMBO J. 25, 910–920
22. Yun, C. C., Sun, H., Wang, D., Rusovici, R., Castleberry, A., Hall, R. A., and Shim, H. (2005) Am. J. Physiol. 289, C2–C11
23. Khundmiri, S. J., Weinman, E. J., Steplock, D., Cole, J., Ahmad, A., Bauern, P. D., Barati, M., Rane, M. J., and Lederer, E. (2005) J. Am. Soc. Nephrol. 16, 2598–25607
24. Kocher, O., Yesilaltay, A., Cirovic, C., Pal, R., Rigotti, A., and Krieger, M. (2003) J. Biol. Chem. 278, 52820–52825
25. Capuano, P., Bacic, D., Stange, G., Hernando, N., Kaissling, B., Pal, R., Kocher, O., Biber, J., Wagner, C. A., and Murer, H. (2005) Pfluegers Arch. Eur. J. Physiol. 449, 392–402
26. Shenolikar, S., Voltz, I. W., Minkoff, C. M., Wade, J. B., and Weinman, E. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 11470–11475
27. Cunningham, R., Steplock, D., Wang, F., Huang, H. E. X., Shenolikar, S., and Weinman, E. J. (2004) J. Biol. Chem. 279, 37815–37821
28. Li, X., Zhang, H., Cheong, A., Leu, S., Chen, Y., Elowsky, C. G., and Donowitz, M. (2004) I. J. Physiol. (Lond.) 556, 791–804
29. McDonough, A. A., and Biemesderfer, D. (2003) Curr. Opin. Nephrol. Hypertens. 12, 533–541
30. Weinman, E. J., Steplock, D., and Shenolikar, S. (2003) FEBS Lett. 536, 141–144
31. Hoogerwerf, W. A., Tsaos, S. C., Devuyst, O., Levine, S. A., Yun, C. H., Yip, J. W., Cohen, M. E., Wilson, P. D., Lazenby, A. J., Tse, C. M., and Donowitz, M. (1996) Am. J. Physiol. 270, G29–G41
32. Collins, J. F., Xu, H., Kiela, P. R., Zeng, J., and Ghishan, F. K. (1997) Am. J. Physiol. 273, C1937–C1946
33. Steinberg, T. H., Newman, A. S., Swanson, J. A., and Silverstein, S. C. (1987) J. Cell Biol. 105, 2695–2702
34. Di Virgilio, F., Steinberg, T. H., Swanson, J. A., and Silverstein, S. C. (1988) J. Immunol. 140, 915–920
35. Chu, S., and Montrose, M. H. (1996) J. Physiol. (Lond.) 494, 783–793
36. Levine, S. A., Nath, S. K., Yun, C. H., Yip, J. W., Montrose, M., Donowitz, M., and Tse, C. M. (1995) J. Biol. Chem. 270, 13716–13725
37. Zizak, M., Lamprecht, G., Steplock, D., Tariq, N., Shenolikar, S., Donowitz, M., Yun, C. H., and Weinman, E. J. (1999) J. Biol. Chem. 274, 24753–24758
38. Morales, F. C., Takahashi, Y., Kreimann, E. L., and Georgescu, M. M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 17705–17710
39. Honegger, K. J., Capuano, P., Winter, C., Bacic, D., Stange, G., Wagner, C. A., Biber, J., Murer, H., and Hernando, N. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 803–808
40. Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T., and Hidaka, H. (1990) J. Biol. Chem. 265, 5267–5272
41. Fujihara, M., Muroi, M., Muroi, Y., Ito, N., and Suzuki, T. (1993) J. Biol. Chem. 268, 14898–14905
42. Enserink, J. M., Christensen, A. E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H. G., Doskeland, S. O., Blank, J. L., and Bos, J. L. (2002) Nat. Cell Biol. 4, 901–906
43. Christensen, A. E., Selheim, F., de Rooij, J., Dremier, S., Schwede, F., Dao, K. K., Martinez, A., Maenhaut, C., Bos, J. L., Genieser, H. G., and Doskeland, S. O. (2003) J. Biol. Chem. 278, 35394–35402
44. Bos, J. L. (2006) Trends Biochem. Sci. 31, 680–686
45. Zhao, J. (2006) Ph.D. thesis, Bos Laboratory.
46. Lamprecht, G., Weinman, E. J., and Yun, C. H. (1998) J. Biol. Chem. 273, 29972–29978
47. Dransfield, D. T., Bradford, A. J., Smith, J., Martin, M., Roy, C., Mangeat, P. H., and Goldenring, J. R. (1997) EMBO J. 16, 35–43