Reciprocal protein kinase A regulatory interactions between cystic fibrosis transmembrane conductance regulator and Na+/H+ exchanger isoform 3 in a renal polarized epithelial cell model

Bagorda, A; Guerra, L; Di Sole, F; Hemle-Kolb, C; Cardone, R A; Fanelli, T; Reshkin, S J; Gisler, S M; Murer, H; Casavola, V

Bagorda, A; Guerra, L; Di Sole, F; Hemle-Kolb, C; Cardone, R A; Fanelli, T; Reshkin, S J; Gisler, S M; Murer, H; Casavola, V. Reciprocal protein kinase A regulatory interactions between cystic fibrosis transmembrane conductance regulator and Na+/H+ exchanger isoform 3 in a renal polarized epithelial cell model. J. Biol. Chem. 2002, 277(24):21480-8.

Postprint available at: http://www.zora.uzh.ch

Posted at the Zurich Open Repository and Archive, University of Zurich.
http://www.zora.uzh.ch

Originally published at: J. Biol. Chem. 2002, 277(24):21480-8.
Reciprocal protein kinase A regulatory interactions between cystic fibrosis transmembrane conductance regulator and Na+/H+ exchanger isoform 3 in a renal polarized epithelial cell model

Abstract

Although Cystic fibrosis transmembrane conductance regulator (CFTR) has been shown to regulate the activity of NHE3, the potential reciprocal interaction of NHE3 to modulate the protein kinase A (PKA)-dependent regulation of CFTR in epithelial cells is still unknown. In the present work, we describe experiments to define the interactions between CFTR and NHE3 with the regulatory, scaffolding protein, NHERF that organize their PKA-dependent regulation in a renal epithelial cell line that expresses endogenous CFTR. The expression of rat NHE3 significantly decreased PKA-dependent activation of CFTR without altering CFTR expression, and this decrease was prevented by mutation of either of the two rat NHE3 PKA target serines to alanine (S552A or S605A). Inhibition of CFTR expression by antisense treatment resulted in an acute decrease in PKA-dependent regulation of NHE3 activity. CFTR, NHE3, and ezrin were recognized by NHERF-2 but not NHERF-1 in glutathione S-transferase pull-down experiments. Ezrin may function as a protein kinase A anchoring protein (AKAP) in this signaling complex, because blocking the binding of PKA to an AKAP by incubation with the S-Ht31 peptide inhibited the PKA-dependent regulation of CFTR in the absence of NHE3. In the A6-NHE3 cells S-Ht31 blocked the PKA regulation of NHE3 whereas it now failed to affect the regulation of CFTR. We conclude that CFTR and NHE3 reciprocally interact via a shared regulatory complex comprised of NHERF-2, ezrin, and PKA.
Reciprocal PKA regulatory interactions between CFTR and NHE3 in a renal polarized epithelial cell model

Names

Bagorda A.*, Guerra L.*, Di Sole F. §, Hemle-Kolb C.§, Cardone R. A., Fanelli T.*, Reshkin S.J.*, Gisler S.M.°, Murer H.° and Casavola V.*

Affiliations.

* Department of General and Environmental Physiology, University of Bari 70126 Bari Italy

§ Department of Physiology and Pathophysiology, Division of Vegetative Physiology and Pathophysiology, Georg-August University of Göttingen D-37073 Göttingen Germany

° Institute of Physiology, University of Zürich CH-8057 Zürich Switzerland

Correspondence to: Valeria Casavola
Department of General and Environmental Physiology
University of Bari
Via Amendola 165/A
70126, Bari, Italy
tel: 39 080 544 3332
fax: 39 080 544 3388
E-mail: casavola@biologia.uniba.it

Running Title: NHERF-2 regulates reciprocal CFTR/NHE3 interaction
Although CFTR has been shown to regulate the activity of NHE3, the potential reciprocal interaction of NHE3 to modulate the PKA-dependent regulation of CFTR in epithelial cells is still unknown. In the present work, we describe experiments to define the interactions between CFTR and NHE3 with the regulatory, scaffolding protein, NHERF that organize their PKA-dependent regulation in a renal epithelial cell line that expresses endogenous CFTR. The expression of rat NHE3 significantly decreased PKA-dependent activation of CFTR without altering CFTR expression and this decrease was prevented by mutation of either of the two rat NHE3 PKA target serines to alanine (S552A or S605A). Inhibition of CFTR expression by antisense treatment resulted in an acute decrease in PKA-dependent regulation of NHE3 activity. CFTR, NHE3 and ezrin were recognized by NHERF-2 but not NHERF-1 in GST pull-down experiments. Ezrin may function as a protein kinase A anchoring protein (AKAP) in this signaling complex since blocking the binding of PKA to an AKAP by incubation with the S-Ht31 peptide inhibited the PKA-dependent regulation of CFTR in the absence of NHE3. In the A6-NHE3 cells S-Ht31 blocked the PKA regulation of NHE3 while it now failed to affect the regulation of CFTR. We conclude that CFTR and NHE3 reciprocally interact via a shared regulatory complex comprised of NHERF-2, ezrin and PKA.
Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP activated Cl⁻ channel expressed in the luminal membrane of secretory and reabsorptive epithelia (1). In addition to transepithelial chloride transport, CFTR has been shown to influence a large number of cell functions including ion transporters such as outwardly rectifying chloride channels, amiloride-sensitive epithelial sodium channels and renal outer medullary potassium channels (2). Initial hypotheses concerning CFTR function suggested that it may function primarily as a global conductance regulator thus magnifying its role in normal cell function (3). Accordingly, defects in CFTR causing the disease cystic fibrosis (CF) lead not only to disturbances of chloride secretion but also of the transport of other electrolytes. In this context, CFTR has been demonstrated to affect both intracellular and extracellular pH regulation by alterations in either HCO₃⁻ secretion via activation and increased expression of the Cl⁻/HCO₃⁻ antiporter (4,5) or by alterations in Na⁺/H⁺ exchanger (NHE) activity (6,7).

The mechanisms by which CFTR exerts its regulatory role over other epithelial ion transporters is still incompletely understood although several hypotheses are currently being explored. These include indirect regulation via release of ATP through CFTR with a subsequent autocrine activation of luminal purinoceptors and modulation of intracellular calcium which in turn could regulate other transporters (8,9). Another regulatory mechanism involves direct intramembrane or cytoplasmic protein-protein interaction. PDZ domain proteins are emerging as important organizing centers for these regulatory complexes and these scaffold-based regulatory proteins are localized to specific sites in polarized epithelial cells. It has been shown that the PKA-dependent regulation of the NHE isoform located in the apical membrane (NHE3) is mediated via the scaffolding protein, NHE-Regulatory Factor (NHERF), whose PDZ2 domain interacts with the cytoplasmic end of NHE3 (10,11). In vitro binding studies have demonstrated that CFTR can bind to the PDZ1 domain of the NHERF with an
intracellular C-terminal domain ending in D-S/T-x-L (12-14) providing a potential mechanism for
the CFTR modulation of the regulation of other membrane proteins such as the NHE3. Recently, using
CFTR and NHE3 cotransfection in fibroblasts, it was demonstrated that CFTR modifies the PKA-
dependent regulation of NHE3 via interaction with the NHERF-1 isoform (7). While some aspects of
the mechanism underlying the CFTR-dependent modulation of NHE3 have been established, the
existence of the potential reciprocal modulation of PKA-dependent regulation of CFTR by NHE3
remains undescribed. Further, as recently discussed (15) the results of many of the studies on regulation
of CFTR have been conducted in non-polarized cells and/or by over-expression of CFTR and, as such,
it may be difficult to draw conclusions about the regulation of CFTR during normal epithelial cell
function.

The objective of the present work was to elucidate the dynamics and mechanism(s) of the
reciprocal alteration of PKA-dependent regulation of CFTR and NHE3 in epithelial cells. To establish
a working model of the dynamic interactions in a polarized epithelial cell, we have used an epithelial
renal cell line, A6, which when grown on permeable filters forms polarized monolayers with a high
transepithelial resistance, an amiloride sensitive sodium transport (16-18), and that also endogenously
expresses wild-type CFTR (19). We have transfected this cell line with: 1) the rat NHE3 (A6-NHE3)
which is functionally expressed on the apical membrane and displays the PKA and PKC regulatory
pattern characteristic for this isoform when endogenously expressed in epithelial cells (20); 2) antisense
oligonucleotides to suppress, transiently, CFTR expression and 3) two mutants of rat NHE3 in which
the PKA target serines 552 or 605 have been mutated to alanine. Using these epithelial cell lines, we
show that 1) the expression of CFTR is necessary for the PKA-dependent regulation of NHE3 while 2)
the expression of NHE3 reduces the PKA-dependent regulation of CFTR; 3) mutation of the PKA
substrate serines 552 or 605 of NHE3 relieves this repression of CFTR regulation; 4) both CFTR and
NHE3 associate with the NHERF-2 and not the NHERF-1 isoform.
Experimental Procedures

Materials

All cell culture materials were purchased from Gibco. BCECF-AM (2’,7’-bis(carboxymethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester) and MQAE (N-[6-methoxyquinoly]acetoxethyl ester) were obtained from Molecular Probes. Glibenclamide and bumetanide were from Sigma. Hygromycin B and nigericin were purchased from Calbiochem. All other chemicals and reagents used were from Sigma or Fluka.

Cell culture

A6/C1 cells used for transfection are a subclone of A6-2F3 cells, functionally selected on the basis of high transepithelial resistance and responsiveness to aldosterone (21). Cell cultures are maintained in 0.8 x concentrated DMEM containing 25 mM NaHCO$_3$, 10% heat-inactivated fetal bovine serum, 50 IU/ml penicillin and 50 mg/ml streptomycin for a final osmolarity of 220-250 mOsmol. Cells were incubated in a humidified 95% air, 5% CO$_2$ atmosphere at 37°C and subcultured weekly by trypsinization using a Ca$^{2+}$/Mg$^{2+}$-free salt solution containing 0.25% (w/v) trypsin and 1 mM EGTA. These cells express endogenous basolateral Na/H exchange activity (22). Transfected cell lines were generated by stable transfection in A6/C1 cells of the cDNAs encoding 1) full length (wild type) ratNHE3 (A6-NHE3), 2) ratNHE3 mutated at single endogenous serine position on the cytoplasmic tail of NHE3 (A6-NHE3S552A and A6-NHE3S605A) and 3) full length OKNHE3 (NHE3 opossum subtype) (A6-NHE3OK). All were subcloned into the pcDNA3.1 vector (Invitrogen, Groningen, Nertherlands) as have been described previously (23). NHE3 constructs all contained a C-terminal
His tag. For transfection, A6 cells were grown to 20-25% confluence in 35 mm tissue culture dishes and DNA was introduced into cells plated on culture dishes using FuGENETM (Boehringer, Mannheim, Germany) and 1.5 µg of the construct of interest together with 0.5 µg of the p3SSLacI vector, which allowed us to select on the basis of hygromycin B resistance (450 µg ml⁻¹ culture medium, for details of the p3SSLacI construct see: (20)). Clonal populations of transfected cell lines obtained by ring cloning were maintained as described above in hygromycin. Cells generally reached confluency between 7 to 8 days after seeding when the culture medium was changed three times a week. Studies on A6 cells were performed between passage 114 to 128. Experiments on A6-NHE3 cells were carried out on cells from passage 22 to 36 while those performed with the PKA deficient mutants were carried out on cells from passage 34 to 38.

Measurement of intracellular pH (pHi) and Na⁺/H⁺-exchange activity

For pH experiments, cells were seeded onto collagen-coated coverslips with a 1.5 mm hole punched in the centre covered by a teflon filter (Millicell-CM, 0.4 µm pore size; Millipore) as described (23). Cells were incubated for 60 min with 5 µM BCECF-AM in Na⁺-medium containing 50 µM probenecid to minimize possible dye leakage. Coverslips with filters containing confluent monolayers were inserted into a chamber that allowed independent perfusion of the apical and basolateral cell surface with Na⁺ medium and placed on the stage of an inverted microscope (Zeiss IM 35). BCECF was excited sequentially by positioning 390-440 nm and 475-490 nm band pass filters in front of a xenon lamp. The emission light was collected by a 515-565 nm band pass filter. pHi was estimated from the ratio of BCECF fluorescence calibrated by using K⁺ nigericin approach (23).
Na\(^{+}\)/H\(^{+}\)-exchange activity was measured by monitoring pHi recovery after an acid load by using the NH\(_4\)Cl prepulse technique (24). The rate of Na\(^{+}\)-dependent alkalinization was determined by linear regression analysis of 15 points taken at 4-sec intervals. The use of nominally CO\(_2\)/HCO\(_3\)^\(-\) free solutions minimizes the likelihood that Na\(^{+}\)-dependent HCO\(_3\)^\(-\) transport was responsible for the observed pHi changes.

Fluorescent measurements of apical chloride efflux

Chloride efflux was measured with the aid of the Cl\(^-\) sensitive dye, MQAE, using the procedure described before (25,26). In brief, A6 and A6-NHE3 cells were seeded onto collagen-coated cell culture inserts having polyethylene terephthalate (PET) filters (Falcon Becton Dickinson). Monolayers were loaded overnight in culture medium containing 5 mM MQAE at 28 °C in a CO\(_2\) incubator. After several washes the filter containing the confluent monolayers were removed from the plastic insert and inserted into a perfusion cuvette that allowed separate superfusion of apical and basolateral cell surfaces (27). Fluorescence was recorded on a Shimadzu RF 5000 spectrofluorometer using 360 nm (bandwidth 10 nm) as excitation wavelength and 450 nm (bandwidth 15 nm) as emission wavelength.

All chloride efflux experiments were performed at room temperature in HEPES-buffered bicarbonate-free media (Cl\(^-\) medium (in mM): NaCl 110, KCl 3, CaCl\(_2\) 1, MgSO\(_4\) 0.5, HEPES 10, KH\(_2\)PO\(_4\) 1, glucose 5 and Cl\(^-\) free medium: NaNO\(_3\) 105, KNO\(_3\) 3.2, MgSO\(_4\) 0.8, NaH\(_2\)PO\(_4\) 1, HEPES 10, CaNO\(_3\) 5, glucose 5). At the start of the experiment the monolayers were perfused at a constant rate of 2 ml/min in the Cl\(^-\) medium. To measure the chloride efflux across the apical
membrane, the apical Cl⁻ perfusion medium was changed to the Cl⁻ free medium and MQAE fluorescence intensity followed. At the end of each experiment a two point calibration procedure was performed: the maximal intensity of fluorescence (F₀) was determined by perfusing the cells with the Cl⁻ free medium on both sides of the monolayer; the minimal fluorescence was obtained by then exposing the cells to a solution containing KSCN (in mM: KSCN 110, MgSO₄ 1, HEPES 10, CaSO₄ 1, glucose 5 and 5 µM valinomycin). For data analysis, the value for minimal fluorescence was subtracted from the experimentally measured fluorescence and the resulting fluorescence divided by the value of F₀. The rate of Cl⁻ efflux was determined by linear regression analysis of 30 points taken at 2 second intervals and expressed in arbitrary slope changes in Δ(F/F₀)/min. To calculate aiCl, a calibration procedure was performed as per Brochiero et al. (26).

Measurements of transepithelial short-circuit current and chloride transport

Measurements of transepithelial potential difference (mV) and short circuit current (µA/cm²) were performed in a modified Ussing chamber according to published methods (28). Transepithelial resistance (Ω x cm²) was calculated according to Ohm’s law. The electrical parameters were measured at room temperature in the following Ringer solution (in mM): NaCl 110, MgSO₄ 0.5, KCl 3, KH₂PO₄ 1, Hepes 10, Glucose 5, CaCl₂ 1 (pH=7.5). As amiloride sensitive sodium channels have been shown in A6 cells to contribute to Isc, chloride transport experiments were conducted in the presence of 10 µM of the sodium channel blocker, amiloride.
Protein extraction and Western blotting.

Total cellular lysates and crude membrane fractions were prepared and their protein content measured as previously described (29). An aliquot of 50 µg protein was separated in 7% SDS-PAGE. The separated proteins were transferred to Immobilon P (Millipore, DuPont) in a Trans-Blot semidry electrophoretic transfer cell (Bio-Rad) for immunoblotting. Immunocomplexes were detected with ECL reagent (Amersham). The following antibody was used: anti CFTR monoclonal antibody against the carboxyl-terminus (R&D Systems, MAB25031, dilution 1:1000). In the cell lysate this antibody recognizes two bands of approximately 175 kDa and 205 kDa (see Figure 3).

Biotinylation of apical membrane proteins

To further characterize the molecular weight and expression levels of the form of CFTR located in the apical membrane, apical cell surface biotinylation experiments were performed. A6 and A6-NHE3 cells were grown on 60-mm Petri dishes and at confluence were washed with ice-cold Ringer NaCl and incubated with 2 mg/ml sulfo-NHS-biotin in Ringer NaCl for 30 min at 4°C. Free sulfo-NHS-biotin was blocked by washing cells twice at 4°C with 0.1 M glycine in Ringer NaCl and then with ice-cold Ringer NaCl. Cells were lysed in buffer lysis (0.4% sodium deoxycholate, 1% Igepal CA-630 (SIGMA), 50 mM EGTA, 10 mM Tris-HCl pH 7.4 plus protease inhibitor cocktails) centrifuged for 10 min (13,000 x g) and the pellet was discarded. 30 µl of Streptavidin-agarose beads was added to the lysates (500 µl) and the mixture was incubated with gentle mixing at 4°C overnight. Streptavidin-bound complexes were pelleted (13,000 x g) and washed three times with 500 µl of lysis buffer. Biotinylated proteins were eluted in Laemmli buffer by boiling for 10 min, resolved by SDS-PAGE, elettroblotted onto Immobilon-P, and immunoblotted with the C-terminal -CFTR antibody (1:1000 dilution).
Pull-down Experiments

The GST-NHERF-1 and GST-NHERF-2 fusion protein homogenates were obtained and the experiments were performed as previously described (29). In brief, equal amounts of GST-NHERF-1 and 2 fusion proteins (≥ 2 µg) were incubated with 25 µl of pre-equilibrated glutathione-agarose beads (Sigma G-4510, 50% slurry) in a total volume of 500 µl of binding buffer (50 mM Tris-HCl, pH 8, 120 mM NaCl, 0.5% Igepal, 5 mM dithiothreitol), by rocking at 4°C for 1 h. After absorption, beads were collected by brief centrifugation at 12,000 rpm for 10 sec (4°C) and gently washed three times with 500 µl of binding buffer containing 0.075% SDS. Pull-down experiments were performed by incubation of these beads with total cellular lysate from A6 and A6-NHE3 cells.

Cellular lysates were prepared from 100 mm-diameter confluent plates. Cells were washed, scraped using 1 ml of binding buffer (50 mM Tris-HCl, pH 8, 120 mM NaCl, 0.5% Igepal-CA-630 and then subjected three times to pulsed sonication for 30 sec on ice. Aliquots were cleared at 12,000 rpm for 2 min (4°C) and ≈ 3 mg protein of these lysates were incubated for 1 h at 4°C with GST-NHERF (1 and 2) immobilized beads. The samples were then washed three times: firstly, with 500 µl of binding buffer without SDS, secondly with 500 µl of binding buffer, diluted 1:2 with same buffer without detergent and, finally, with 500 µl of binding buffer further diluted 1:2. The resulting pellet was extracted in Laemmly buffer and used for SDS-PAGE electrophoresis. Western blotting was performed with either the monoclonal anti-human CFTR antibody directed against the C-terminus or a monoclonal anti-human Ezrin antibody (BD Transduction Laboratories) or the anti-rat NHE3 antibody 1568 (gift of Prof. O. W. Moe, Univ. Texas) obtained as described (30), and immunoreactive bands were detected by ECL using a secondary HRP-coupled IgG (Sigma A-8924).

Antisense Oligodeoxynucleotide (ODNs) Treatment
A 21 mer phosphorothioate-modified antisense (AS) ODN complementary to the 5’ end of the open reading frame of X. laevis CFTR was produced (5’-CTCCAGCGGCGTCTTCTGCAT-3’). A corresponding missense (MS) ODN sequence (5’-ACGCTGGCTCTACGTTCGCTC-3’) was used as control. ODNs, phosphorothioated for stability on the first 5 and last 5 nucleotides and purified by HSFP, were purchased from MWG Biotech. A synthetic cationic lipid (Lipofectamine, Boehringer Mannheim) was used to increase uptake and stability of the ODNs. AS or MS ODNs were added to the complete culture medium of cell monolayers at final concentrations of 10 µM ODN and 13 µM Lipofectamine for 5 hrs, at which time the growth medium was replaced. At 48 hrs post-treatment, cells were collected for Western Blot analysis of CFTR expression and the cells were assayed for transport activity.

Data presentation

Results are presented as mean ± SE. Statistical comparisons were made using the paired and unpaired data Student’s t test and p< <0.05 was considered statistically significant.
Results

A6 cells, a cell line derived from the distal part of the nephron of the toad (Xenopus laevis), are known to have transporters for both electrogenic sodium uptake and for electrogenic chloride secretion. Patch-clamp experiments in A6 cells have demonstrated the presence of two types of apical Cl⁻ channels which are controlled by calcium and/or cAMP (17). In addition, Ling et al. (19) have demonstrated the existence of the chloride channel, CFTR, on their apical membrane.

We first validated the functional presence of CFTR on the apical membrane of the A6 cells and the basic mechanism of its PKA-dependent up-regulation. CFTR activity was measured by two parallel and independent techniques: via measurements of 1) transepithelial short circuit current, Isc, or 2) the efflux of chloride across the apical membrane measured by changes in fluorescence of the chloride sensitive dye, MQAE. A6 cells grown on permeable filters formed a polarized monolayer displaying a high transepithelial resistance (8400 ± 600 Ω cm², n=22). In all Isc experiments, amiloride (10 µM) was present in the apical bath solution to inhibit electrogenic Na⁺ absorption. Under these conditions cAMP stimulated Isc is referable to Cl⁻ secretion. Figure 1 shows that the basolateral application of 10 µM forskolin (FSK) elicited a rapid increase in the Isc. The subsequent apical addition of glibenclamide, a well known inhibitor of CFTR (31), significantly inhibited the FSK-dependent Isc increase (-53.5 ± 4.6%, n=6, p<0.01). On the other hand, DIDS (100 µM), a stilbene derivative known to inhibit other anion transporters but not CFTR (32), did not inhibit the forskolin-stimulated Isc (-0.71 ± 20.1%, n=5, n.s.). Further support for the hypothesis that FSK induced Cl⁻ secretion was derived from experiments in which the response to FSK was examined during perfusion of both sides of the epithelium with Cl⁻-free Ringer solution. Substitution of chloride with nitrate strongly reduced
the FSK-dependent Isc increase obtained in the presence of chloride by 88.9 ± 0.5% (n=3). In the histogram reported in Figure 1, glibenclamide-sensitive chloride transport (ie. CFTR-mediated chloride transport) is indicated as the clear bar and was calculated as the difference of FSK-stimulated Isc in the absence (light gray bar) and presence (dark bar) of glibenclamide.

Figure 2 illustrates a typical experiment and the summary of 9 experiments of apical chloride efflux. After substitution of chloride by nitrate in the apical perfusion medium (see experimental procedures), the rate of chloride efflux was measured by the change in fluorescence ($\Delta(F/F_0)/\text{min}$) of the chloride sensitive dye, MQAE, as described (25). As also observed in the same cell line by Banderali et al. (25), a small amount of basal Cl$$^\text{-}$$ efflux occurred under baseline conditions when the chloride solution was replaced by the nitrate solution (0.025 ± 0.007 $\Delta(F/F_0)/\text{min}$, n=9). Subsequent FSK (10 µM) treatment of the same monolayer increased the chloride efflux rate and glibenclamide added apically before the next stimulation with FSK significantly inhibited this chloride transport. In the histogram of Figure 2, the clear bar represents the glibenclamide-sensitive Cl$$^\text{-}$$ efflux (CFTR-mediated chloride transport) calculated from the difference in alterations of forskolin-stimulated fluorescence measurement in the absence (light gray bar) and presence (dark bar) of glibenclamide.

The Na$$^+$/K$$^+$/2Cl$$^\text{-}$$ cotransporter, which is responsible for chloride loading of the cells, has been demonstrated to be stimulated by cAMP (33). In the experiments summarized in Figure 2, we treated the basolateral side of the monolayer with bumetanide (5 µM) for five minutes before each stimulation with FSK to avoid the possibility that the observed increase of chloride efflux induced by FSK could be due in part to the stimulation of the Na$$^+$/K$$^+$/2Cl$$^\text{-}$$ cotransporter. However, the glibenclamide-sensitive chloride transport obtained in presence of bumetanide was not significantly different from that obtained
In a parallel series of experiments in which we did not pretreat the cells with bumetanide (glibenclamide sensitive Cl− efflux: 0.0202 ± 0.002 (F/F₀)/min, n=7 vs 0.0169 ± 0.002 (F/F₀)/min, n=9 n.s, in the absence or presence of bumetanide, respectively).

Influence of NHE3 on CFTR activity

The ability of active CFTR to influence the PKA-dependent regulation of NHE3 activity in fibroblasts co-transfected with CFTR and NHE3 via coupled interaction of the two transporters with NHERF has been recently reported (7) and, thus, it could be hypothesized that NHE3 can affect CFTR regulation. We, therefore, next examined whether there exists the reciprocal modulation by NHE3 on PKA-dependent regulation of CFTR activity. To accomplish this, we utilized A6 cells stably transfected with cDNA encoding the rat subtype of NHE3 (A6-NHE3 cells). As previously reported, the transfected NHE3 is expressed on the apical membrane and is inhibited (-38.14 ± 2.72, n=6 p<0.001) by stimulation of PKA with forskolin (20).

We first determined if expressing NHE3 alters the expression of CFTR. As can be seen in a typical Western Blot (Figure 3A), two bands of approximately 175 kDa (arrowhead) and 205 kDa (arrow) were observed in the total cellular lysate while only the 205 kDa band was observed in either a membrane fraction or in the streptavidin-precipitate of the biotinylated apical cell surface proteins. To demonstrate that the anti-C-terminus antibody utilized in the experiments is specific for the CFTR in A6 cells, we immunoprecipitated CFTR from cell lysate as described by Ling et al. (19) with a different antibody (against the regulatory domain, R&D Systems, MAB1660) and Western analysis performed with the above carboxyl-terminus antibody recognized the same two proteins (Figure 3B). These data would suggest that the 175 kDa band could represent the immature, intracellular form and the 205 kDa
band represents the mature CFTR. In order to further validate that the upper band corresponds to mature CFTR, we deglycosilated crude membrane fractions with either Endoglycosidase H or N-Glycosidase F since mature CFTR has been demonstrated to be resistant to Endoglycosidase H deglycosilation but sensitive to N-Glycosidase F deglycosylation (34) and we observed that the band was indeed unaffected by Endoglycosidase H and reduced in molecular weight by N-Glycosidase F treatment (Fig. 3C). Further data to support this hypothesis came from experiments in which expression of only the 205 kDa band was reduced by blocking the ER-to-Golgi traffic with 1µg/ml brefeldin A (data not shown), a treatment that blocks the maturation of CFTR (35). As can be seen in Figure 3A, the expression of NHE3 did not affect the expression of either the mature CFTR (arrow) or of the immature, non-glycosylated CFTR (arrowhead). CFTR band intensity in A6-NHE3 cells was 95 ± 4% of that in A6 cells in blots quantitated by densitometric analysis., n=7, n.s.).

We next examined the effect of NHE3 expression on the PKA-regulated activity of CFTR by comparing the effect of FSK (10 µM) on both the amiloride sensitive Isc (chloride secretion) in the absence or presence of glibenclamide (Fig. 4A) and the glibenclamide-sensitive chloride efflux across the apical membrane measured by changes in fluorescence of the chloride sensitive dye, MQAE (Fig. 4B). It can be seen that in both types of measurements the apical expression of NHE3 in A6-NHE3 cells reduced the glibenclamide-sensitive chloride efflux by approximately 60%. The basal chloride efflux in A6-NHE3 cells was not significantly different from that measured in A6 cells (0.0164 ± 0.005 (F/F0)/min, n=8 in A6-NHE3 vs 0.025 ± 0.007 Δ(F/F0)/min, n=9 in A6; n.s) and importantly, there was no difference in intracellular chloride concentration between A6 and A6-NHE3 cells loaded with MQAE (24.5 ± 2.4 vs 27.7 ± 4.1 mM, in A6, n=18, and A6-NHE3, n=11, cells, respectively).

Moreover, the expression of NHE3 on the apical membrane raised the resting pHi from 7.22 ±
0.09 (n=8) in A6 cells to 7.64 ± 0.09 (n=6) in A6-NHE3 cells (p < 0.01). Cellular alkalinization has been shown to favour the stimulation of CFTR by cAMP (36) suggesting that it is not the effect of NHE3 activity on cytosolic pH that mediates its observed negative effect on forskolin-stimulation of CFTR in the A6-NHE3 cells. Altogether, these data clearly indicate that it is the presence of functional NHE3 that negatively modulates (ie. decreases) the PKA-dependent regulation of CFTR.

In order to evaluate if the site of integration of the exogenous DNA for rat NHE3 could account for the observed ‘interaction’ between NHE3 and CFTR, we transfected A6 cells with another subtype (opossum) of the NHE3 isoform (A6-NHE3OK) (37). Indeed, the negative modulation of PKA-dependent regulation of CFTR activity by the presence of NHE3 was confirmed in this cell line: neither the amount of PKA-dependent inhibition of NHE3 activity (45.24 ± 2.65%, n=4, A6-NHE3OK vs 38.14 ± 2.72%, n=6, in A6-NHE3 cells, respectively) nor the value of the CFTR-mediated Cl⁻ efflux (0.0086 ± 0.009, n=6 in A6-NHE3OK vs 0.0078 ± 0.006 (F/F₀)/min, n=7, in A6-NHE3) were significantly different in A6 cells transfected with the opossum or rat subtype of the NHE3 isoform. These data further support that the presence of functional NHE3 negatively modulates (ie. decreases) the regulation of CFTR activity by PKA.

While the mechanism by which PKA phosphorylation of NHE3 leads to its inhibition is still unknown, it is clear that this phosphorylation is necessary for its functional regulation (38). A critical question in the context of the current study is if the PKA-dependent phosphorylation of NHE3 is necessary for its modulation of PKA-induced CFTR regulation. To this purpose, we analyzed the CFTR-dependent apical secretion in transfected A6 cells with rat NHE3 in which either of the PKA target serines 552 or 605 was mutated to alanine (37). Previous studies, in fact, have demonstrated that these two serines are PKA phosphorylation substrates in rat NHE3 and are necessary for its PKA
dependent regulation (38, 39, 40). We found that both the S552A and S605A mutations completely reversed the FSK inhibitory effect on NHE3 activity (respectively in the absence and presence of FSK: 0.275±0.04 vs 0.265±0.4 pHi/min in A6-NHE3S552A, n=6 n.s and 0.394±0.046 vs 0.402±0.05 pHi/min in A6-NHE3S605A, n=4, n.s.). As illustrated in Figure 5A, the expression of membrane CFTR did not change in any of these transfected cell lines. The cell monolayers with the mutated NHE3 were then stimulated with forskolin and CFTR-dependent apical chloride efflux analyzed by fluorescence measurements as in Figure 2. Figure 5B shows that when PKA can no longer phosphorylate either of these two serines, the regulation of CFTR activity by forskolin returned to levels not significantly different from that observed in the wild-type A6 cells. These results demonstrate that endogenous NHE3 phosphorylation by PKA is an absolute requirement for its modulation of PKA-dependent regulation of CFTR. Altogether, these data imply that the PKA-dependent regulation of CFTR but not its expression is negatively modulated by functional NHE3.

Inhibition of CFTR expression results in a decrease of the PKA-dependent regulation of apical NHE3 activity.

As we found a negative modulating effect of NHE3 expression on PKA-dependent regulation of CFTR activity, we felt it necessary to validate the positive effect of CFTR on NHE3 regulation that was previously demonstrated in fibroblasts (7) in the A6-NHE3 cells which express CFTR endogenously on the apical membrane. Inhibition of CFTR expression by an antisense oligonucleotide (ODN) against the CFTR start site has been previously utilized in cells expressing endogenous CFTR to demonstrate that CFTR modulates the activity and regulation of the ENaC sodium channels (19). Based on these observations, we synthetized a 21 mer antisense ODN or its missense against the
Xenopus laevis CFTR start site in order to confirm the occurrence and pattern of modulation of the PKA-dependent regulation of apical NHE3 activity by endogenous, apically located CFTR. Incubation of A6-NHE3 cells with 10 µM of antisense for 48 hrs led to a marked reduction of both CFTR protein expression (Fig. 6A) and of the forskolin-dependent stimulation of CFTR activity (the mean reduction of the CFTR chloride efflux by antisense treatment was -49.4 ± 2.8%, n=3, p< 0.02). This is a slightly higher level of inhibition of CFTR activity by the antisense ODN treatment than that reported by Ling et al. (19).

We then determined the effect of this missense and antisense treatment on the PKA-dependent regulation of the apical NHE3 in the A6-NHE3 cell line. As can be seen in Figure 6B, this antisense-induced reduction in CFTR expression had no effect on basal transfected, apical NHE3 activities (controls, solid bars) but resulted in an almost complete loss of the forskolin-dependent regulation of the NHE3 activity while, in the missense transfected cells (MS-CFTR), forskolin inhibited the NHE3 to almost the same level as in the non-transfected cells. These results confirm the reported modulatory effect of CFTR on NHE3 regulation by PKA in a highly polarized cell line that endogenously expresses CFTR.

Role of NHERF-2 in the reciprocal PKA-dependent regulation of CFTR and NHE3 activity.

It has been demonstrated that both NHE3 and CFTR can associate with either the NHERF-1 (11,41) or the NHERF-2 (14) isoform. In the renal cortical collecting duct the NHERF-2 isoform is more strongly expressed (42), suggesting that in the A6 cells NHERF-2 could be the relevant isoform. We therefore examined, via GST-fusion protein pull-down assays, the association of these two NHERF isoforms with CFTR and NHE3 in the A6 and A6-NHE3 cell lines. Figure 7 shows that, indeed, CFTR (Fig. 7A) was pulled-down from total cellular lysate by NHERF-2 but not NHERF-1 in
both cell lines. It is noteworthy that, although both mature and immature CFTR are present in the lysate (see Fig. 3), only the immature form was pulled-down by NHERF-2 (see arrowhead). Similar experiments performed in A6-NHE3OK cells confirmed that the low molecular weight, immature form of CFTR associates preferentially with NHERF-2 (data not shown).

As can seen in Fig. 7B, NHE3 was pulled-down by only NHERF-2 and only in the A6-NHE3 cell line. In brush border membrane fractions from rat kidney, used as positive controls, NHE3 was also recognized almost exclusively by the NHERF-2 fusion protein.

The NHERF directed PKA phosphorylation of target proteins has been demonstrated to be mediated by the association of the AKAP protein, ezrin, to NHERF in a wide variety of cell contexts (14,43). In order to determine if ezrin forms a part of this signaling complex in our cell model, we probed NHERF-1 and NHERF-2 pull-downs with an anti-ezrin antibody. Indeed, as can be seen in Figure 7C, ezrin was found to associate with the two NHERF isoforms in both cell lines.

Role of the AKAP protein ezrin in the reciprocal PKA-dependent regulation of CFTR and NHE3 activity.

Recent work has demonstrated that A-kinase anchoring proteins (AKAPs) play a fundamental role in governing the cellular compartmentalization of PKA in order to localize it in proximity of the target substrate. This appears to be due to the binding of the regulatory PKA subunits RII to the AKAPs at a specific amino acid consensus sequence that can be blocked by the synthetic peptide Ht31 (44). Recently, it has been demonstrated that PKA associates with CFTR by the AKAP protein, ezrin (45). To determine if PKA-dependent regulation of CFTR is mediated by anchorage to an AKAP in our cell models and how this is altered by the presence of NHE3, we preincubated monolayers of either A6 or
A6-NHE3 cells for 30 minutes with Ht31, an amphipathic peptide that corresponds to the RII binding motif of a human thyroid AKAP (46) or with its inert analog, Ht31-P containing prolines at positions 502 and 507 (47) and measured the PKA-dependent stimulation of CFTR-dependent chloride efflux in both cell lines and inhibition of NHE3 in the A6-NHE3 cell line. We have used Ht31 and Ht31-P coupled to stearate residues (S-Ht31 and S-Ht31-P) and thus rendered membrane-permeable (47). As can be seen in Figure 7, preincubation with S-Ht31 almost completely eliminated forskolin-dependent stimulation of apical CFTR-dependent chloride transport in A6 cells. In contrast, S-Ht31 treatment had no effect on the CFTR-dependent efflux in the A6-NHE3 cells (Fig.8) while it completely prevented the PKA-dependent inhibition of NHE3 (Fig. 9). The inert analogue, S-Ht31-P, had no effect on the PKA-dependent regulation of either CFTR (Fig. 8) or NHE3 (Fig. 9) transport activity.

The fact that Ht31 did not affect forskolin-mediated activation of CFTR in A6-NHE3 cells suggests that when NHE3 is coexpressed with CFTR the protein complex ezrin-NHERF2 might anchor the Type II regulatory subunits of PKA (PKA II) in proximity to NHE3. In this way the residual forskolin activation of CFTR could regulated predominantly by the cytosolic, non-anchored Type I PKA. Singh et al. (48) have reported that CFTR can be regulated by both Type I and II PKA in T84 cells. In support of this hypothesis, we found that 10 µM of the pan-specific PKA inhibitor, H89, was able to almost completely inhibit the CFTR-dependent chloride efflux in A6-NHE3 cells by 86 ± 6% (n=4, p<0.001).
Discussion

In addition to transepithelial chloride transport, CFTR has been shown to influence a large number of cell functions including the transport of other electrolytes (3). By influencing these electrolyte transports it appears that CFTR plays a fundamental role in regulating cell content and volume of fluids. As a modulator of transepithelial sodium transport, CFTR has been demonstrated to play a modulating role in the PKA-dependent regulation of the activity of both sodium channel (ENaC) (49) and Na⁺/H⁺ exchanger isoform 3 (NHE3) (7). The mechanism underlying the PKA-dependent regulation of NHE3 involves direct interaction with PDZ-containing scaffolding proteins such as the NHERF in which the NHERF can function to link NHE3 with ezrin, a protein kinase A anchoring protein, creating a multi-protein complex and, thereby, mediating the PKA-dependent regulation of NHE3 (10,43,50). There is evidence demonstrating that either NHERF isoform can also associate with CFTR via the PDZ1 domain (12,41) to confer, via direct, ezrin-mediated phosphorylation, PKA-dependent regulation of its activity (14).

This co-ordination of PKA-dependent regulation of either NHE3 or CFTR in the apical plasma membrane of epithelia by NHERF suggests a mechanism by which CFTR could regulate the NHE3 through the joint association with NHERF. Indeed, it has been recently demonstrated, by co-transfection of NHE3 and CFTR in fibroblasts, that CFTR modifies the PKA-dependent regulation of NHE3 via interaction with the NHERF-1 isoform (7). Conversely, the joint interaction of CFTR and NHE3 with NHERF suggests the existence of a reverse, reciprocal modulating effect of NHE3 on CFTR regulation. Along this line, recent work examining the relationship between CFTR and ENaC sodium channels demonstrated such a reciprocal interaction between CFTR and ENaC: CFTR not only acts as a regulator of ENaC but is, in turn, regulated by ENaC (51).
In the present study, we have considered the possibility of the existence of a potential reciprocal modulation of PKA-dependent regulation of CFTR by NHE3. In order to study this reciprocal interaction between CFTR and NHE3 in a highly polarized monolayer, we used a cell line expressing an endogenous CFTR (19) and a transfected rat NHE3 on the apical membrane (20). CFTR and ezrin associated with NHERF-2 in both cell lines and NHE3 associated with NHERF-2 in the A6-NHE3 cell line (Fig. 7). In these cell lines, we verified that in CFTR antisense-treated A6-NHE3 monolayers, forskolin was no longer able to inhibit the NHE3 activity. These data confirm that CFTR is required for the PKA-dependent inhibition of Na⁺ absorption driven by NHE3 as reported in both heterologous double transfected fibroblasts (7) and in mouse intestine (52) and support the hypothesis that CFTR and NHE3 could interact via a common regulatory scaffold protein.

The most significant finding of the present study was that the PKA-dependent regulation of CFTR is also, in turn, negatively modulated by the presence and activity of NHE3. The PKA-dependent regulation of CFTR-mediated Cl⁻ secretion was lower in A6-NHE3 than in A6 cells without a change in either CFTR protein expression (Fig. 3) or association of CFTR with NHERF-2 (Fig. 7). The same pattern of modulation of the PKA-dependent regulation of CFTR-mediated Cl⁻ secretion by NHE3 expression and association of CFTR with NHERF-2 was observed in an A6 cell line that had been stably transfected with the opossum subtype of NHE3 (A6-NHE3OK).

The mutation of either of the two PKA phosphorylation substrate serines, 552 or 605, to alanine (40) prevented the forskolin-induced inhibition of NHE3 and significantly relieved the negative modulating effect of NHE3 on PKA-dependent regulation of CFTR activity without a change in CFTR expression (Fig. 5), demonstrating that it is not the presence of NHE3 but the phosphorylation by PKA that is required for the negative influence of NHE3 on CFTR regulation. All together, the data suggest
either that NHE3 has a higher affinity for associating with the regulatory NHERF-ezrin-PKA complex or that CFTR functions to direct PKA-dependent regulation to NHE3 when the two transporters are functionally co-expressed. That is, when only CFTR is expressed it is the substrate for the NHERF-ezrin-PKA complex while when NHE3 is co-expressed, CFTR becomes a component in a new complex for NHE3 regulation as was previously suggested (7). This could explain how CFTR can function either as a PKA-regulated chloride channel or as a transmembrane regulatory protein for other transporters: it is the relative expression of the various components of this regulatory module that determines which function CFTR will have. A significant confirmation for this hypothesis came from the experiments in which we pretreated A6 and A6-NHE3 cells with S-Ht31 which prevents the binding between AKAPs and Type II regulatory subunits of PKA (47). Indeed, S-Ht31 interfered with the PKA-mediated activation of CFTR in A6 cells, suggesting that the PKA-CFTR interaction is mediated by an AKAP as recently was observed in Calu-3 airway cells (45). In the A6-NHE3 cells, S-Ht31 completely blocked the forskolin-mediated inhibition of NHE3 activity (Fig. 9) while it was no longer able to block the activation of CFTR chloride efflux by forskolin (Fig. 8), suggesting that the co-expression of NHE3 led to the targeting of the multiprotein complex ezrin-PKAII-NHERF-2 to the proximity of NHE3 rather than to CFTR.

A possible complementary mechanism for this last hypothesis could be that an interaction between CFTR and both of the two PDZ domains of NHERF-2 is needed for the PKA-dependent increase in CFTR activity. Recently, Raghuram et al. (53) demonstrated that NHERF binds to the cytoplasmic tail of CFTR through either of its two PDZ domains although with a higher affinity for the PDZ1 domain. The association of CFTR with both domains regulated channel gating by crosslinking the C-terminal tails in a single dimeric CFTR channel which resulted in an increase in the open probability. In this model, when NHE3 binds to one of the PDZ domains of NHERF, CFTR is no
longer able to be in the dimeric form and thus loses part of its PKA-dependent regulation. A configuration of dimeric CFTR would give rise to more active channels when activated by PKA, thus modulation of the intermolecular CFTR interaction is an attractive mechanism for the potentiation of its activity by PKA (54). The same mechanism was recently demonstrated for the adapter protein, CAP70 (55), in which the CFTR channel is switched to a more active conductive state via an interaction with the two CAP70 PDZ domains, suggesting that this mechanism is widespread.

In conclusion, the novel finding of this study was that there is a reciprocal interaction between CFTR and NHE3 for PKA-dependent regulation. To determine the precise mechanism whereby NHE3 co-expression influences the regulation of CFTR by PKA will require further investigation. Thus, NHE3, like ENaC, is not simply a passive recipient of CFTR regulatory action but plays an active role by altering, in turn, CFTR function.

Acknowledgements

A.B. and L.G. contributed equally to this work. We thank Dr. E. Klussman of the Free University, Berlin, Germany for generously providing the S-Ht31 and S-Ht31-P peptides and Prof. O.W. Moe of the Univ. Of Texas, Dallas, Texas for the gift of the anti-NHE3 antibody. This work was funded by grant E.1125 of the Italian Telethon.
Figure Legends

Figure 1. A) Typical time course of short-circuit current (Isc) and transepithelial potential difference (ΔV) during stimulation with 10 μM basolateral forskolin (FSK) in the absence or presence of apical glibenclamide (300 μM). The measurements were always conducted in the presence of 10 μM amiloride to block the apical sodium channels. The increase of FSK-dependent Isc was significantly diminished by glibenclamide applied on the apical side. B) Summary of this inhibitory effect in a series of 6 experiments. The empty bar represents the CFTR-dependent component of the transepithelial Isc calculated as the difference of the amiloride-independent, forskolin-stimulated increase in Isc in the absence of (light gray bar) and presence of (dark bar) glibenclamide. Each bar represents the mean ± S.E. of paired measurements.

Figure 2. A) Typical recording showing changes in intracellular Cl⁻-dependent MQAE fluorescence (expressed as the F/F₀ ratio) when the A6 cell monolayer was treated with 10 μM FSK following substitution of apical chloride by nitrate in the absence or presence of glibenclamide (300 μM). Glibenclamide was applied for five minutes before apical anion substitution. The basolateral side was perfused with a chloride solution containing 5 μM bumetanide for 5 minutes before each stimulation with FSK to block the cAMP sensitive basolateral Na⁺/K⁺/2Cl⁻ cotransporter. B) Summary of these data from 9 independent experiments where the glibenclamide-sensitive Cl⁻ efflux rates across the apical membrane (empty bar) were calculated as the difference in the F/F₀ ratio per minute ((F/F₀)/min) in the absence of (light grey bar) and presence of (dark bar) glibenclamide. Each bar represents the mean ± S.E.. In the same monolayer we first followed the rate of Cl⁻ efflux after forskolin treatment and then always in the same monolayer we analyzed the effect of glibenclamide; this permitted the use of two-tailed, paired Students t-test analysis of the data.

Figure 3. Stable expression of rat NHE3 has no effect on CFTR expression. A) Total cellular lysates, crude membrane fractions or a streptavidin-precipitate of biotinylated apical membrane proteins were prepared from A6 and A6-NHE3 cells, separated by 7% SDS-PAGE and blotted onto Immobilon-P for Western Blot analysis with monoclonal antibody against the CFTR C-terminus. The position of the
mature, glycosylated and the immature, core form of CFTR are indicated by an arrow and an arrowhead, respectively. B) CFTR was immunoprecipitated from A6 cell lysate with an antibody against the regulatory domain (R&D Systems MAB1660) and Western analysis performed with an antibody against its carboxyl-terminus (R&D Systems MAB 25031). C) Crude membrane fractions from A6 and A6-NHE3 cells were digested as described in Experimental Procedures with either Endoglycosidase H or N-Glycosidase F.

Figure 4. Stable expression of rat NHE3 decreases cAMP-mediated activation of CFTR chloride transport. A) The glibenclamide-sensitive components of the forskolin-stimulated, amiloride-insensitive Isc in A6 and A6-NHE3 cells were calculated as in Figure 1. Data are mean ± S.E. for 6 and 4 unpaired experiments, respectively. B) The glibenclamide-sensitive Cl⁻ efflux rates in A6 and A6-NHE3 cells were calculated as in Figure 2. Data are mean ± S.E. for 9 and 7 unpaired experiments, respectively.

Figure 5. PKA-dependent phosphorylation of NHE3 is necessary for modulation of cAMP-mediated activation of CFTR chloride transport. A) Expression of CFTR in crude membrane extracts from all four cell lines measured by Western Blot as in Figure 3. B) The glibenclamide-sensitive chloride efflux was measured as in Figure 2 in A6, A6-NHE3, A6-NHE3S552A and A6-NHE3S605A cell lines. Data are mean ± S.E. of 9, 7, 4 and 7 unpaired experiments, respectively.

Figure 6. CFTR antisense but not missense treatment reduced CFTR expression and prevented cAMP-mediated inhibition of NHE3 activity in A6-NHE3 cells. Monolayers were transfected with 10 µM antisense or missense ODN complementary to the 5' end open reading frame start site of X. laevis CFTR and, 48 hrs later, the expression of CFTR and measurement of NHE3 activity measured. Antisense but not missense treatment A) depleted the expression of CFTR in whole cell lysates measured by Western Blotting as in Figure 3 and B) led to a reversal of the forskolin-dependent inhibition of apical NHE3 activity measured by monitoring changes in fluorescence of the pH sensitive dye, BCECF, as described in Experimental Procedures. Data are mean ± S.E. for 6, 5 and 4 paired experiments for control, missense and antisense, respectively.
Figure 7. CFTR and NHE3 are recognized by NHERF-2 but not NHERF-1 in GST pull-down. Total cell lysates from A6 and A6-NHE3 cells were prepared and pull-down experiments conducted as described in Experimental Procedures. A) A typical Western Blot for CFTR is shown in which lanes 1 and 2 are total cellular lysates, lane 3 is A6 cell lysate incubated with GST-alone, lanes 4 and 5 are extracts of the GST-NHERF-1 pellet of A6 and A6-NHE3 lysates and lanes 6 and 7 are extracts of the GST-NHERF-2 pellet of A6 and A6-NHE3 lysates. As in Figure 3, the position of the mature, glycosylated and the immature, core form of CFTR are indicated by an arrow and an arrowhead, respectively. B) An analogous experiment in which the blot was probed with a polyclonal antibody against rat NHE3. As positive control, the GST-NHERF-1 and GST-NHERF-2 pull-down measurements were performed with brush border membrane derived from rat kidney. C) An analogous experiment in which the blot was probed with a monoclonal antibody against ezrin.

Figure 8. The synthetic AKAP blocking peptide, S-Ht31, inhibits PKA-dependent stimulation of CFTR chloride efflux in A6 but not A6-NHE3 cells. Cell monolayers were preincubated for 30 min with 100 µM of the anti-AKAP peptide S-Ht31 or its inactive form S-Ht31-P and the effect of FSK (10 µM) on CFTR activity was analyzed as in Figure 2. Data are mean ± S.E. for 9, 5 and 5 unpaired experiments for control, S-Ht31-P and S-Ht31 in A6 cells and 7, 3 and 5 unpaired experiments for control, S-Ht31-P and S-Ht31 in A6-NHE3 cells, respectively.

Figure 9. The synthetic AKAP blocking peptide, S-Ht31, inhibits PKA-dependent inhibition of NHE3 activity in A6-NHE3 cells. Cell monolayers were preincubated for 30 min with 100 µM of the anti-AKAP peptide S-Ht31 or its inactive form S-Ht31-P and the effect of FSK (10 µM) on NHE3 activity was analyzed as in Figure 5B. Data are mean ± S.E. for 7, 5 and 6 experiments for control, S-Ht31-P and S-Ht31 experiments, respectively. Experiments reported in this figure were carried out such that control pHi recovery was performed first and then always compared to the pHi recovery after the treatment performed on the same monolayer. This permitted the use of two-tailed, paired Students t-test analysis of the data.
References

1. Sheppard, D. N., and Welsh, M. J. (1999) *Physiol Rev* 79(1 Suppl), S23-45.
2. Schwiebert, E. M., Benos, D. J., Egan, M. E., Stutts, M. J., and Guggino, W. B. (1999) *Physiol Rev* 79(1 Suppl), S145-66.
3. Quinton, P. M. (1999) *Physiol Rev* 79(1 Suppl), S3-S22.
4. Lee, M. G., Wigley, W. C., Zeng, W., Noel, L. E., Marino, C. R., Thomas, P. J., and Muallem, S. (1999) *J Biol Chem* 274(6), 3414-21.
5. Wheat, V. J., Shumaker, H., Burnham, C., Shull, G. E., Yankaskas, J. R., and Soleimani, M. (2000) *Am J Physiol Cell Physiol* 279(1), C62-71.
6. Casavola, V., Turner, R. J., Guay-Broder, C., Jacobson, K. A., Eidelman, O., and Pollard, H. B. (1995) *Am J Physiol* 269(1 Pt 1), C226-33.
7. Ahn, W., Kim, K. H., Lee, J. A., Kim, J. Y., Choi, J. Y., Moe, O. W., Milgram, S. L., Muallem, S., and Lee, M. G. (2001) *J Biol Chem* 276(20), 17236-43.
8. Schwiebert, E. M., Egan, M. E., Hwang, T. H., Fulmer, S. B., Allen, S. S., Allen, S. S., Cutting, G. R., and Guggino, W. B. (1995) *Cell* 81(7), 1063-73.
9. Walsh, D. E., Harvey, B. J., and Urbach, V. (2000) *J Membr Biol* 177(3), 209-19.
10. Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J., and Lefkowitz, R. J. (1998) *Proc Natl Acad Sci U S A* 95(15), 8496-501.
11. Moyer, B. D., Duhaime, M., Shaw, C., Denton, J., Reynolds, D., Karlson, K. H., Pfeiffer, J., Wang, S., Mickle, J. E., Milewski, M., Cutting, G. R., Guggino, W. B., Li, M., and Stanton, B. A. (2000) *J Biol Chem* 275(35), 27069-74.
12. Sun, F., Hug, M. J., Lewarchik, C. M., Yun, C. H., Bradbury, N. A., and Frizzell, R. A. (2000) *J Biol Chem* 275(38), 29539-46.
13. Chalfant, M. L., Coupaye-Gerard, B., and Kleyman, T. R. (1993) *J Gen Physiol* 101(10), 2199-206.
14. Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J., and Lefkowitz, R. J. (1998) *Proc Natl Acad Sci U S A* 95(15), 8496-501.
15. Moyer, B. D., Duhaime, M., Shaw, C., Denton, J., Reynolds, D., Karlson, K. H., Pfeiffer, J., Wang, S., Mickle, J. E., Milewski, M., Cutting, G. R., Guggino, W. B., Li, M., and Stanton, B. A. (2000) *J Biol Chem* 275(35), 27069-74.
16. Sun, F., Hug, M. J., Lewarchik, C. M., Yun, C. H., Bradbury, N. A., and Frizzell, R. A. (2000) *J Biol Chem* 275(38), 29539-46.
17. Widdicombe, J. H. (2000) *Am J Respir Cell Mol Biol* 22(1), 11-4.
18. Chalfant, M. L., Coupaye-Gerard, B., and Kleyman, T. R. (1993) *J Am Physiol* 264(6 Pt 1), C1480-8.
19. Marunaka, Y., and Eaton, D. C. (1991) *Am J Physiol* 260(5 Pt 1), C1071-84.
20. Kleyman, T. R., Ernst, S. A., and Coupaye-Gerard, B. (1994) *Am J Physiol* 266(3 Pt 2), F506-11.
21. Ling, B. N., Zuckerman, J. B., Lin, C., Harte, B. J., McNulty, K. A., Smith, P. R., Gomez, L. M., Worrell, R. T., Eaton, D. C., and Kleyman, T. R. (1997) *J Biol Chem* 272(1), 594-600.
22. Di Sole, F., Casavola, V., Mastroberardino, L., Verrey, F., Moe, O. W., Burckhardt, G., Murer, H., and Helmle-Kolb, C. (1999) *J Physiol* 515(Pt 3), 829-42.
23. Verrey, F. (1994) *J Membr Biol* 138(1), 65-76.
24. Casavola, V., Guerra, L., Reshkin, S. J., Jacobson, K. A., and Murer, H. (1997) *Mol Pharmacol* 51(3), 516-23.
25. Casavola, V., Reshkin, S. J., Murer, H., and Helmle-Kolb, C. (1992) *Pflugers Arch* 420(3-4), 282-9.
26. Boron, W. F., and De Weer, P. (1976) *J Gen Physiol* 67(1), 91-112.
25. Banderali, U., Brochiero, E., Lindenthal, S., Raschi, C., Bogliolo, S., and Ehrenfeld, J. (1999) J Physiol 519 Pt 3, 737-51.
26. Brochiero, E., Banderali, U., Lindenthal, S., Raschi, C., and Ehrenfeld, J. (1995) Pflugers Arch 431(1), 32-45.
27. Krayer-Pawlowska, D., Helme-Kolb, C., Montrose, M. H., Krapf, R., and Murer, H. (1991) J Membr Biol 120(2), 173-83.
28. Casavola, V., Guerra, L., Reshkin, S. J., Jacobson, K. A., Verrey, F., and Murer, H. (1996) J Membr Biol 151(3), 237-45.
29. Casavola, V., Guerra, L., Reshkin, S. J., Jacobson, K. A., Verrey, F., and Murer, H. (1996) J Membr Biol 151(3), 237-45.
30. Amemiya, M., Loffing, J., Lotscher, M., Kaissling, B., Alpern, R. J., and Moe, O. W. (1995) Kidney Int 48(4), 1206-15.
31. Schultz, B. D., Singh, A. K., Devor, D. C., and Bridges, R. J. (1999) Physiol Rev 79(1 Suppl), S109-44.
32. Schwiebert, E. M., Flotte, T., Cutting, G. R., and Guggino, W. B. (1994) Am J Physiol 266(5 Pt 1), C167-S173.
33. Kopito, R. R. (1999) Physiol Rev 79, S167-S173.
34. Reddy, M. M., Light, M. J., and Quinton, P. M. (1999) Nature 402(6759), 301-4.
35. Di Sole F., C. R., Moe O.W., Burckdardt G. and Helmle-Kolb C. (2001) FASEB Journal 15(4), p.A144
36. Weinman, E. J., Steplock, D., Donowitz, M., Shenolikar, S., and Weinman, E. J. (2000) Biochemistry 39(20), 6123-9.
37. Colledge, M., and Scott, J. D. (1999) Trends Cell Biol 9(6), 216-21.
38. Sun, F., Hug, M. J., Bradbury, N. A., and Frizzell, R. A. (2000) J Biol Chem 275(19), 14360-6.
39. Carr, D. W., Hausken, Z. E., Fraser, I. D. C., Stofko-Hahn, R. E., and Scott, J. D. (1992) J Biol Chem 277(8), 4934-8.
40. Klussmann, E., Maric, K., Wiesner, B., Beyermann, M., and Rosenthal, W. (1999) J Biol Chem 274(8), 4934-8.
41. Singh, A. K., Tasken, K., Walker, W., Frizzell, R. A., Watkins, S. C., Bridges, R. J., and Bradbury, N. A. (1998) Am J Physiol 275(2 Pt 1), C562-70.
42. Kunzelmann, K., Schreiber, R., Nitschke, R., and Mall, M. (2000) Pflugers Arch 440(2), 193-201.
43. Dransfield, D. T., Bradford, A. J., Smith, J., Martin, M., Roy, C., Mangeat, P. H., and Goldenring, J. R. (1997) Embo J 16(1), 35-43.
44. Jiang, Q., Li, J., Dubroff, R., Ahn, Y. J., Foskett, J. K., Engelhardt, J., and Kleyman, T. R.
(2000) J Biol Chem 275(18), 13266-74.

52. Clarke, L. L., and Harline, M. C. (1996) Am J Physiol 270(2 Pt 1), G259-67.
53. Raghuram, V., Mak, D. D., and Fossett, J. K. (2001) Proc Natl Acad Sci U S A 98(3), 1300-5.
54. Anderson, M. P., Sheppard, D. N., Berger, H. A., and Welsh, M. J. (1992) Am J Physiol 263(1 Pt 1), L1-14.
55. Wang, S., Yue, H., Derin, R. B., Guggino, W. B., and Li, M. (2000) Cell 103(1), 169-79.
