Acute Regulation of Na/H Exchanger NHE3 by Adenosine A1 Receptors Is Mediated by Calcineurin Homologous Protein

Received for publication, June 26, 2003, and in revised form, October 6, 2003
Published, JBC Papers in Press, October 21, 2003, DOI 10.1074/jbc.M306838200

Francesca Di Sole‡§§, Robert Cerulli†, Victor Babich†, Henry Quiñones‡, Serge M. Gisler‡‡, Jürg Biber†, Heini Murer‡, Gerhard Burckhardt‡, Corinna Helmle-Kolb§**, and Orson W. Moe‡**‡‡§§

From the ‡Department of Internal Medicine and §§Center of Mineral Metabolism and Clinical Research, Medical Center, University of Texas Southwestern, Dallas, Texas 75390-8856, the ¶§Medical Service, Veterans Affairs Medical Center, Dallas, Texas 75216, the †Department of Physiology and Pathophysiology, Georg-August-University of Göttingen, Göttingen 37073, Germany, and the ¶¶Institute of Physiology, University of Zürich, Zürich 8057, Switzerland

Adenosine is an autacoid that regulates renal Na+ transport. Activation of adenosine A1 receptor (A1R) by N'-cyclopentidyladenosine (CPA) inhibits the Na+/H+ exchanger 3 (NHE3) via phospholipase C/Ca2+/protein kinase C (PKC) signaling pathway. Mutation of PKC phosphorylation sites on NHE3 does not affect regulation of NHE3 by CPA, but amino acid residues 462 and 552 are essential for A1R-dependent control of NHE3 activity. One binding partner of the NHE family is calcineurin homologous protein (CHP). We tested the role of NHE3-CHP interaction in mediating CPA-induced inhibition of NHE3 in opossum kidney (OK) and Xenopus laevis uroepithelial (A6) cells. Both native and transfected NHE3 and CHP are present in the same immunocomplex by co-immunoprecipitation. CPA (10−6 M) increases CHP-NHE3 interaction by 30–60% (native and transfected proteins). Direct CHP-NHE3 interaction is evident by yeast two-hybrid assay (bait, NHE3C terminus; prey, CHP); the minimal interacting region is localized to the juxtamembrane region of NHE3C terminus (amino acids 462–552 of opossum NHE3). The yeast data were confirmed in OK cells where truncated NHE3 (NHE3Δ552) still shows CPA-stimulated CHP interaction. Overexpression of the polypeptide from the CHP binding region (NHE3Δ552) interferes with the ability of CPA to inhibit NHE3 activity and to increase CHP-NHE3 full-length interaction. Reduction of native CHP expression by small interference RNA ablates the ability of CPA to inhibit NHE3 activity. We conclude that CHP-NHE3 interaction is regulated by A1R activation and this interaction is a necessary and integral part of the signaling pathway between adenosine and NHE3.

Adenosine is an ubiquitous nucleoside generated from AMP by 5'-nucleotidase or from S-adenosyl-L-homocysteine (SAH)1 by SAH hydrolase (1, 2). Renal adenosine is derived exogenously from the systemic circulation through mainly neurovascular sources or endogenously via the intrarenal AMP and SAH pathways (2). Adenosine modulates many functions in mammals via binding to A1, A2A, A2B, and/or A3 adenosine receptors (1, 3). In the kidney, adenosine regulates glomerular filtration rate, renin release, erythropoietin production, cellular proliferation, and tubular water and Na+ transport (2, 4).

Renal adenosine generation is markedly increased in response to hypoxia, ischemia, or inflammation (2, 3). It is well documented that adenosine can exert its protective effect against acute renal ischemia by containing inflammatory damage inflicted by circulating immune cells (2, 3). A concomitant protective effect during ischemia can be achieved by reducing oxygen expenditure of the renal tubules through inhibition of Na+ transport, the principal oxygen-consuming process (5, 6). In the renal proximal tubules, which constitute over 70% of renal cortical mass, adenosine decreases the activity of the Na+/H+ exchanger NHE3 on the apical membrane of the proximal tubule (7–9). The inhibition of apical Na+ entry decreases the activity of the Na+/K+–ATPase and oxygen requirement of the highly aerobic proximal tubule (10, 11).

NHE3 belongs to a superfamily of electroneutral mammalian Na+/H+ exchangers that has eight members documented to date (12–17). With the exception of NHE5, all NHE isoforms are found in the kidney. All NHEs have a predicted N-terminal hydrophobic ion-translocating domain and a variable C-termini
dominal hydrophilic domain that harbors regulatory sequences (15–18). NHE3 is the isoform in the apical membrane based on antigenic (19, 20) and functional data (21–24). Non-NHE3-mediated Na+/H+ exchange activity in the proximal tubule apical membrane (24) can theoretically be caused by NHE8 (14), but definitive data are still forthcoming. The current study focuses on NHE3.

Using a heterologous system, we found that acute A1R and A2R stimulation inhibits NHE3 via primarily PKC-coupled and PKA-coupled pathways, respectively (7). In a recent report (8), we showed that early A1R activation is associated with a change in intrinsic transport activity of NHE3, whereas sus-

** This work was supported in part by the National Institutes of Health Grants R01-DK-54892 and R01-DK-54992 (to O. W. M.), by American Heart Association Texas Affiliate Grant 98G-052 (to O. W. M.), by the Department of Veteran Affairs Research Service (to O. W. M.), by National Institutes of Health National Research Service Award T32 DK07257-2031 (to H. Q.), by the Deutsche Forschungsgemeinschaft (Grant HE 2418/2–1 to C. H.-K.), and by the Swiss National Science Foundation (Grants 31-46523.96 and 31-65397.01 to H. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of American Heart Association Texas award (0325098Y).

‡ To whom correspondence should be addressed: Dept. of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-8856. Tel.: 214-648-3152; Fax: 214-648-2071; e-mail: Francesca.DiSole@UTsouthwestern.edu.

§§ Both authors contributed equally to the work.

** Both authors contributed equally to the work.

Preparation of this manuscript was supported in part by the National Institutes of Health Grants 31-46523.96 and 31-65397.01 to H. M., by the Deutsche Forschungsgemeinschaft (Grant HE 2418/2–1 to C. H.-K.), and by the Swiss National Science Foundation (Grants 31-46523.96 and 31-65397.01 to H. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†† Recipient of American Heart Association Texas award (0325098Y).

‡‡ To whom correspondence should be addressed: Dept. of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-8856. Tel.: 214-648-3152; Fax: 214-648-2071; e-mail: Francesca.DiSole@UTsouthwestern.edu.

§§§ Both authors contributed equally to the work.

‡‡‡ Both authors contributed equally to the work.

‡‡‡‡ Both authors contributed equally to the work.

¶¶¶¶ Both authors contributed equally to the work.

1 The abbreviations used are: SAH, S-adenosyl-L-homocysteine; NHE3, Na+/H+ exchanger 3; A1R, adenosine A1 receptor; A2R, adenosine A2 receptor; CPA, protein kinase A; PKC, protein kinase C; CHP, calcineurin homologous protein; OK, opossum kidney cells; EGFP, enhanced green fluorescent protein; HA, hemagglutinin; aa, amino acids; CMV, cytomegalovirus; TMA, tetratramethylammonium; PBS, phosphate-buffered saline; siRNA, small interference RNA.
Regulation of NHE3 by Adenosine Is Mediated by CHP

**FIG. 1.** Effect of the A1R agonist CPA on native and transfected NHE activities in OK cells and A6 cells. NHE activity was assayed microfluorometrically as the rate of Na\(^+\)/H\(^+\) exchange activity in response to A1R stimulation from the apical or basolateral site. In A6 cells where A1R are located in the apical membrane, CPA (10\(^{-6}\) M) was applied to the apical cell surface for 15 or 30 min as indicated. In OK cells stimulation of A1R was performed between passages 23 and 57. A6/C1 cells are a subclone of OK cells selected on the basis of expression of parathyroid hormone-sensitive Na\(^+\)/phosphate co-transport (32) and transfected NHE3. A6/C1 and transfected A6 cell lines were grown in 0.8–1.2 cm \(^2\) wells in a humidified 95%/5% air/CO\(_2\) atmosphere at 37\(^\circ\)C and sub-cultured weekly. Cells generally reached confluence within 3–4 days, and experiments were conducted 1–2 days after confluence. Studies on OK cells were performed between passages 23 and 57. A6/C1 cells are a subclone of A6-2F3 cells, functionally selected on the basis of high transepithelial resistance and responsiveness to aldosterone (33). These cells express endogenous basolateral Na\(^+\)/H\(^+\) exchange activity (7, 34) and transfected NHE3 is targeted to the apical membrane (7, 8). A6/C1 cell lines were grown in 0.8× concentrated Dulbecco’s modified Eagle’s medium, supplemented with 25 mM NaHCO\(_3\), 10% heat-inactivated fetal bovine serum, 50 IU ml\(^{-1}\) penicillin and 50 \(\mu\)g ml\(^{-1}\) streptomycin in a humidified 95%/5% air/CO\(_2\) atmosphere at 37 \(^\circ\)C and sub-cultured weekly. Cells generally reached confluence within 3–4 days, and experiments were conducted 1–2 days after confluence. Studies on OK cells were performed between passages 23 and 57. A6/C1 cells are a subclone of A6-2F3 cells, functionally selected on the basis of high transepithelial resistance and responsiveness to aldosterone (33). These cells express endogenous basolateral Na\(^+\)/H\(^+\) exchange activity (7, 34) and transfected NHE3 is targeted to the apical membrane (7, 8).

**EXPERIMENTAL PROCEDURES**

Cell Culture and Transfection—Experiments were performed on OK and A6/C1 cells. A subclone of OK cells selected on the basis of expression of parathyroid hormone-sensitive Na\(^+\)/phosphate co-transport (32) was cultured in a mixture of Dulbecco’s modified Eagle’s medium Ham’s F-12 (Invitrogen, Carlsbad CA) supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 IU ml\(^{-1}\) penicillin and 50 \(\mu\)g ml\(^{-1}\) streptomycin in a humidified 95%/5% air/CO\(_2\) atmosphere at 37 \(^\circ\)C and sub-cultured weekly. Cells generally reached confluence within 3–4 days, and experiments were conducted 1–2 days after confluence. Studies on OK cells were performed between passages 23 and 57. A6/C1 cells are a subclone of A6-2F3 cells, functionally selected on the basis of high transepithelial resistance and responsiveness to aldosterone (33). These cells express endogenous basolateral Na\(^+\)/H\(^+\) exchange activity (7, 34) and transfected NHE3 is targeted to the apical membrane (7, 8).

**Fig. 1.** Effect of the A1R agonist CPA on native and transfected NHE activities in OK cells and A6 cells. NHE activity was assayed microfluorometrically as the rate of Na\(^+\)/H\(^+\) exchange activity in response to A1R stimulation from the apical or basolateral site. In A6 cells where A1R are located in the apical membrane, CPA (10\(^{-6}\) M) was applied to the apical cell surface for 15 or 30 min as indicated. In OK cells stimulation of A1R was performed between passages 23 and 57. A6/C1 cells are a subclone of OK cells selected on the basis of expression of parathyroid hormone-sensitive Na\(^+\)/phosphate co-transport (32) and transfected NHE3. A6/C1 and transfected A6 cell lines were grown in 0.8–1.2 cm \(^2\) wells in a humidified 95%/5% air/CO\(_2\) atmosphere at 37\(^\circ\)C and sub-cultured weekly. Cells generally reached confluence within 3–4 days, and experiments were conducted 1–2 days after confluence. Studies on OK cells were performed between passages 23 and 57. A6/C1 cells are a subclone of A6-2F3 cells, functionally selected on the basis of high transepithelial resistance and responsiveness to aldosterone (33). These cells express endogenous basolateral Na\(^+\)/H\(^+\) exchange activity (7, 34) and transfected NHE3 is targeted to the apical membrane (7, 8). A6/C1 cell lines were grown in 0.8× concentrated Dulbecco’s modified Eagle’s medium, supplemented with 25 mM NaHCO\(_3\), 10% heat-inactivated fetal bovine serum, 50 IU ml\(^{-1}\) penicillin and 50 \(\mu\)g ml\(^{-1}\) streptomycin in a humidified 95%/5% air/CO\(_2\) atmosphere at 37\(^\circ\)C. For A6 cells transfected with NHE3, medium was supplemented with 450 \(\mu\)g ml\(^{-1}\) (pH) recovery. CPA (10\(^{-6}\) M) was applied to the apical cell surface for 15 or 30 min as indicated. In OK cells stimulation of A1R was from the apical or basolateral site. In A6 cells where A1R are located in the apical membrane, CPA was added only to the apical cell surface. Data are expressed as percentage of activity of untreated cells. The number of experiments, each consisting of a pair of control and agonist-treated cells, is given in parentheses. Bars and error bars represent means and standard errors, respectively. Asterisks indicate statistical significance from control measurements (*, p < 0.05; **, p < 0.01, ANOVA). A, effect of CPA on apical NHE3 activity in opossum kidney (OK) cells. B, effect of CPA on opossum NHE3 stably transfected into A6 cells. C, effect of CPA on rat NHE3 stably transfected into A6 cells. D, effect of CPA on endogenous basolateral Xenopus NHE (XNHE) in untransfected (A6/C1) and transfected A6 cell lines.
Regulation of NHE3 by Adenosine Is Mediated by CHP

Figu 2. Effect of CPA on membrane NHE3 protein abundance in OK cells. CPA (10−6 M for 15 or 30 min) or vehicle-treated cells were labeled with biotin on cell surface, and biotinylated proteins were precipitated with streptavidin-bioglue agarose. NHE3 abundance was measured by immunoblot using anti-OK-NHE3 (9683) antibodies. Total cellular NHE3 protein abundance was measured on cell lysate by immunoblot. A, representative blot. B, summary of all experiments. Number of experiments is given in parentheses, and significance from control measurements is indicated by asterisks (*, p < 0.05, ANOVA). C, OK cells were transiently transfected with the NHE3/eGFP. Forty-eight hours after transfection, cells were visualized by fluorescent microscopy prior to and after exposure to CPA for 15 or 30 min.

Ecoli. Cells generally reached confluence between 7 and 8 days after seeding, and studies were performed between passages 6 and 43.

For transient transfection, cells were grown to ~70% confluence in culture dishes, and 1.3 µg of cDNA was introduced into cells using LipofectAMINE (Invitrogen). Transfection efficiency was monitored by co-transfection of the pEGFP plasmid (Clontech, Palo Alto, CA) and was approximately 70%. For stable transfection, A6 cells were grown to 20–25% confluence in 35-mm tissue culture dishes, and cDNA was introduced into cells using FuGENE 6 (Roche Applied Science, Mannheim, Germany). The construct of interest (1.5 µg) was co-transfected with the p35SSLac vector (0.5 µg) to allow selection by hygromycin B resistance (7). Clonal populations of transfected cell lines obtained by ring cloning were maintained in hygromycin.

Plasmid Constructs—Generation of constructs encoding for wild-type and mutated forms of rat-NHE3 (at either a single endogenous serine position or at six endogenous serine positions on the cytoplasmic tail of NHE3) has been previously described (8, 36, 37). Full-length and truncated forms of NHE3 cDNAs were introduced into cells using LipofectAMINE with forward and reverse primers flankned with the appropriate restriction endonuclease sites and sub-cloned into the desired plasmids. All constructs were verified by direct sequencing. Mammalian expression plasmids and inserts included: 1) C-terminal c-Myc and hexahistidine (His6)-tagged rat and opossum NHE3 (NHE3/myc/6H) (pcDNA3.1, Invitrogen, Carlsbad, CA); 2) C-terminal human influenza hemagglutinin (HA)-tagged mouse-CHP (CHP/HA) (pMHTM, Roche Applied Science, Indianapolis, IN); 3) C-terminal enhanced green fluorescent protein (eGFP)-tagged OK-NHE3 (NHE3/eGFP) (Clontech, Palo Alto, CA); and 4) N-terminal FLAG-tagged OK-NHE3 sequence comprising aa 462–552 (FLAG/NHE3/462–552) (pFLAG-CMV2TM-2, Sigma, St. Louis, MO). For yeast two-hybrid bait construction, the C terminus of mouse NHE3 was cloned from mouse kidney poly-A+ RNA using primers derived from rat NHE3 (forward 5'-ggcattcccgagttgaaagc-3'; reverse 5'-gggtggtgactcatgtggactcag-3') using low stringency PCR. After sequence verification, EcoRI and Sal restriction sites were introduced by PCR into cDNA fragments polymerized from wild-type mouse NHE3 (mNHE3, terminus) as template. Bait constructs (NHE3794–839 (aa 382–839), NHE3802–839 (aa 382–839), NHE3794–794 (aa 382–794), NHE3802–557 (aa 382–557), NHE3802–501 (aa 382–501), NHE3766–839 (aa 766–839), and NHE3802–839 (aa 769–839)) were made as in-frame fusion proteins with the LexA DNA binding domain in the vector pBTM116 carrying the TRP1 selection marker (38) (Clontech).

Measurement of Na+/H+ Exchange Activity—NHE activity was measured as the rate of Na+-dependent recovery of cytosolic pH (pHj) following an acid load (NH4Cl prepulse) as described previously (8). Confluent cells on permeable support (collagen-coated coverslip with a 1.5-mm central perforation covered by a Millicell-CM 0.4-µm Teflon filter (Millipore, Eschborn, Germany) were dye-loaded (4.2 µM 2,7'-bis(2-carboxyethyl)-5-(and -6)-carboxyfluorescein, acetoxymethyl ester; 50 µM probenecid) in Na+-free medium (60 min) and superfused continuously with Na+-medium on the stage of an inverted microscope (Zeiss Axiovert 100). After recording baseline pH, cells were acid-loaded (Na+ medium + 40 mM NH4Cl, followed by Na+ -free medium) and subsequent addition of Na+ medium yielded NHE activity as the rate of pHj recovery. In A6 transfectants, addition of Na+ medium to either the apical or basolateral cell surface allowed independent polarized measurement of NHE activity. Media used in pH measurements were nominally bicarbonate-free. For OK cells: isotonic Na+-containing media used for experiments were treated for 6–7 days with 1 µM dexamethasone known to accelerate maturation and differentiation (35). To ensure true segregation of the apical and basolateral surface, we established A6/C1 cell lines exhibited reproducible high transepithelial resistance. Untransfected A6/C1 cells transepithelial resistance was comparable with NHE3-transfected ones (data not shown).

Plasmid Constructs—Generation of constructs encoding for wild-type and mutated forms of rat-NHE3 (at either a single endogenous serine position or at six endogenous serine positions on the cytoplasmic tail of NHE3) has been previously described (8, 36, 37). Full-length and truncated forms of NHE3 cDNAs were introduced into cells using LipofectAMINE with forward and reverse primers flankned with the appropriate restriction endonuclease sites and sub-cloned into the desired plasmids. All constructs were verified by direct sequencing. Mammalian expression plasmids and inserts included: 1) C-terminal c-Myc and hexahistidine (His6)-tagged rat and opossum NHE3 (NHE3/myc/6H) (pcDNA3.1, Invitrogen, Carlsbad, CA); 2) C-terminal human influenza hemagglutinin (HA)-tagged mouse-CHP (CHP/HA) (pMHTM, Roche Applied Science, Indianapolis, IN); 3) C-terminal enhanced green fluorescent protein (eGFP)-tagged OK-NHE3 (NHE3/eGFP) (Clontech, Palo Alto, CA); and 4) N-terminal FLAG-tagged OK-NHE3 sequence comprising aa 462–552 (FLAG/NHE3/462–552) (pFLAG-CMV2TM-2, Sigma, St. Louis, MO). For yeast two-hybrid bait construction, the C terminus of mouse NHE3 was cloned from mouse kidney poly-A+ RNA using primers derived from rat NHE3 (forward 5'-ggcattcccgagttgaaagc-3'; reverse 5'-gggtggtgactcatgtggactcag-3') using low stringency PCR. After sequence verification, EcoRI and Sal restriction sites were introduced by PCR into cDNA fragments polymerized from wild-type mouse NHE3 (mNHE3, terminus) as template. Bait constructs (NHE3794–839 (aa 382–839), NHE3802–839 (aa 382–839), NHE3794–794 (aa 382–794), NHE3802–557 (aa 382–557), NHE3802–501 (aa 382–501), NHE3766–839 (aa 766–839), and NHE3802–839 (aa 769–839)) were made as in-frame fusion proteins with the LexA DNA binding domain in the vector pBTM116 carrying the TRP1 selection marker (38) (Clontech).

Measurement of Na+/H+ Exchange Activity—NHE activity was measured as the rate of Na+-dependent recovery of cytosolic pH (pHj) following an acid load (NH4Cl prepulse) as described previously (8). Confluent cells on permeable support (collagen-coated coverslip with a 1.5-mm central perforation covered by a Millicell-CM 0.4-µm Teflon filter (Millipore, Eschborn, Germany) were dye-loaded (4.2 µM 2,7'-bis(2-carboxyethyl)-5-(and -6)-carboxyfluorescein, acetoxymethyl ester; 50 µM probenecid) in Na+-free medium (60 min) and superfused continuously with Na+-medium on the stage of an inverted microscope (Zeiss Axiovert 100). After recording baseline pH, cells were acid-loaded (Na+ medium + 40 mM NH4Cl, followed by Na+ -free medium) and subsequent addition of Na+ medium yielded NHE activity as the rate of pHj recovery. In A6 transfectants, addition of Na+ medium to either the apical or basolateral cell surface allowed independent polarized measurement of NHE activity. Media used in pH measurements were nominally bicarbonate-free. For OK cells: isotonic Na+-containing media contained (in mM): 130 NaCl, 4 KCl, 1 CaCl2, 1 MgSO4, 1 NaH2PO4, 18
glucose, and 20 HEPES buffered to pH 7.4 with NaOH; isotonic TMA+ medium composed of (in mM): 130 tetramethylammonium chloride (TMACl), 4 KCl, 1 CaCl2, 0.5 MgSO4, 1 K2HPO4, 5 glucose, and 20 HEPES, pH 7.4, with TMAOH. For A6 cells: isotonic Na+ medium contained (in mM): 110 NaCl, 3 KCl, 1 CaCl2, 0.5 MgSO4, 1 KH2PO4, 5 glucose, and 10 HEPES, pH 7.5, with Tris; isotonic TMA+ medium contained (in mM): 110 TMACl, 3 KCl, 1 CaCl2, 0.5 MgSO4, 1 KH2PO4, 5 glucose, and 10 HEPES, pH 7.5, with Tris. The microfluorometry was performed on a Zeiss Axiosvert 100 inverted microscope with a Zeiss LD “Achroplan” 63×/0.75 objective, coupled to a multichannel illumination system (Polychrome II from T.I.L.L. Photonics GmbH, Grafelfing, Germany). A monochromatizing device with an integral light source (55-watt xenon arc lamp) alternatively selected the excitation wavelengths (495 ± 10 and 440 ± 10 nm) via a galvanometric scanner and directed to the cells via a 515-nm dichroic mirror and a 535 ± 25-nm band-pass collected filter fluorescence emission. Photometric data were acquired using the MetaFlour software (Visitron Systems, Puchheim, Germany). Calibration of the fluorescence signals to pH, cells were performed in the presence of the K+ ionophore nigericin (0.5 μM) in isotonic K+–rich medium as described previously (39). For A6 cells the isotonic K+–rich medium used contained (in mM): 105 KCl, 8 NaCl, 1 CaCl2, 0.5 MgSO4, 1 KH2PO4, 5 glucose, and 10 HEPES buffered to various pH values for calibration of intracellular BCECF.

Quantification of Surface NHE3 Antigen—The assay was performed as described previously (40). OK cells were grown to confluence on 100-mm culture dishes, whereas A6 cells stably expressing wild-type OK-NHE3 were grown on 40-cm² collagen-coated Nucleopore Polycarbonate Track-Etch membranes (Fischer Scientific, Hampton, NH). The latter configuration allowed polarized quantification of NHE3 expression in the apical versus basolateral membranes. After exposure to agonists or vehicle, cells were rinsed in Ca/Mg/PBS (in mM: 150 NaCl, 10 Na2HPO4, pH 7.4, 0.1 CaCl2, 1 MgCl2), incubated with the arginine- and lysine-reactive NHS-SS-biotin (2 mg ml⁻¹, Pierce, Rockford, IL) in buffer (in mM: 150 NaCl, 10 triethanolamine, pH 7.4, 2 CaCl2) for 1 h. After quenching in Ca/Mg/PBS supplemented with 100 mM glycine, cells were lysed in biotin-RIPA buffer (in mM: 150 NaCl, 50 Tris-HCl, pH 7.4, 5 mM EDTA, 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS) and centrifuged (109,000 g, 50,000 rpm, 25 min, 4 °C, Beckman TLX ultracentrifuge, TLA 100.3 rotor, Beckman, Fullerton, CA), and the protein content in the supernatant was quantified by the Bradford method (Bio-Rad, Hercules, CA). Equal protein amounts of cell lysate were equilibrated with streptavidin-agarose beads at 4 °C, washed sequentially with solution A (in mM: 50 Tris-HCl, pH 7.4, 100 NaCl, 5 EDTA), solution B (in mM: 50 Tris-HCl, pH 7.4, 500 NaCl), and solution C (in mM: 50 Tris-HCl, pH 7.4). Biotinylated proteins were released by incubation in 100 mM dithiothreitol, reconstituted in Laemmli buffer, and subjected to SDS-PAGE and blotted with anti-OK-NHE3 antisera (#5683) (40). The integrity of the monolayer was confirmed by measurement of trans-epithelial resistance prior to the experiment and again following surface labeling with NHS-SS-biotin.

Yeast Two-hybrid—Bait vectors were transformed into Saccharomyces cerevisiae (strain L40) containing the genotype MATa trp1 leu2 his3 LYS2::LexA-HIS3 URA3::LexA-LacZ (38, 41). Yeast cells were grown in YPD medium (1% yeast extract, 2% Difco peptone, 2% glucose, 0.003% adenine hemisulfate) or the synthetic minimal Trp dropout medium (SD-Trp) complemented with 0.005% adenine hemisulfate (42). For transformation, a YPD culture from L40 (20 ml) grown to an A600 of

![Image](image-url)
A cDNA library (MATCHMAKER, Clontech) of whole adult mouse kidneys was screened as previously described (44) except mNHE3 cytosol was used as bait. cDNA inserts were ligated to the C terminus of the GAL4 activation domain in vector pACT2, which delivers the leu2 nutritional gene for complementation. Putative positive colonies were isolated by consecutive colony lift assays where the permeabilized cells transferred onto Whatman filters were overlaid with 0.2 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Alexis, Lausen, Switzerland), 150 mM NaCl, 50 mMTris-HCl (pH 7.4), and 0.8% agarose (45, 46). Yeast DNA of true positives was isolated (47), and prey plasmids were rescued by transformation into KC8 cells, which carry trpC, leuB, and hisB mutations (48). Positivity of purified preys was validated once.

FIG. 4. CPA- or PMA-induced modulation of the activity wild-type NHE3 and selected mutated forms of rat NHE3 stably transfected into A6 cells and comparison to regulation of native basolateral Xenopus NHE (XNHE) activity. Na+/H+ exchange activity was measured microfluorometrically as Na+-induced alkalization (independent apical versus basolateral Na+ addition) after an acid load (NH4Cl pulse/withdrawal). Pair-wise comparisons of NHE activity were made before and after addition of either apical CPA (10⁻⁶ M) or PMA (10⁻⁷ M). The number of experiments performed under identical experimental conditions is given in parentheses. Bars and error bars represent means and standard errors, respectively. Asterisks indicate significant difference from control measurements (*, p < 0.05; **, p < 0.01, ANOVA). A, summary of effect of agonists on transfected NHE3 activities. B, summary of effect of agonists on native basolateral Xenopus NHE (XNHE) activity in untransfected (A6/C1) and transfected cell lines. A6-ratNHE3, A6 cells stably transfected with wild-type rat NHE3; A6-ratNHE3S605G, A6 cells stably transfected with mutant NHE3 (substitution of serine residue in position 605 by glycine); A6-ratNHE3S605G, A6 cells stably transfected with mutant NHE3 carrying a multitude of six point mutations (substitution of serine residues in positions S513G, S552A, S575A, S661A, S690G, and S804G by either glycine (G) or alanine (A) as indicated).

~1 (18 h) was centrifuged at 1,200 × g for 3 min, washed with 40 ml of distilled H2O, and resuspended in 1 ml of distilled H2O. Aliquots of 50 µl of competent yeast were treated with 240 µl of polyethylene glycol (50% w/v), 36 µl of 1 M LiAc, 25 µl of single-stranded DNA (2.0 mg/ml), 50 µl of distilled H2O, and 1 µg of the plasmid of interest, according to Gietz and Schiestl (43). After an incubation at 30°C (30 min at 260 rpm) followed by a heat shock at 42°C (20 min), cells were harvested at 16,500 rpm for 15 s in a tabletop centrifuge, resuspended in 100 µl of distilled H2O, and plated onto 14-cm SD-Trp Petri dishes.

A dDNA library (MATCHMAKER, Clontech) of whole adult mouse kidneys was screened as previously described (44) except mNHE3 was used as bait. dDNA inserts were ligated to the C terminus of the GAL4 activation domain in vector pACT2, which delivers the leu2 nutritional gene for complementation. Putative positive colonies were isolated by consecutive colony lift assays where the permeabilized cells transferred onto Whatman filters were overlaid with 0.2 mg/ml 5-bro-

FIG. 5. Effect of CPA or PMA on truncated versions of opossum NHE3 stably expressed in A6 cells. A, illustration of full-length and truncation mutant forms of NHE3. The hydrophobic membrane-spanning terminus of NHE3 is shown as a dark bar, whereas the portion of the hydrophilic C terminus is shown as an open bar, with the amino acid position at the truncation site indicated for each mutant. B and C, effect of apical CPA (10⁻⁶ M, 15 min) or PMA (10⁻⁷ M, 15 min) on NHE activity measured microfluorometrically as rate of Na⁺ dependent intracellular pH (pHᵢ) recovery.

FIG. 5. Effect of CPA or PMA on truncated versions of opossum NHE3 stably expressed in A6 cells. A, illustration of full-length and truncation mutant forms of NHE3. B and C, effect of apical CPA (10⁻⁶ M, 15 min) or PMA (10⁻⁷ M, 15 min) on NHE activity measured microfluorometrically as rate of Na⁺ dependent intracellular pH (pHᵢ) recovery. The number of experiments, each consisting of a pair of control and agonist-treated cells, is given in parentheses. Bars and error bars represent means and standard errors, respectively. Asterisks indicate significant difference from control measurements (*, p < 0.05; **, p < 0.01, ANOVA). NHE3 mut, cells transfected with truncated versions of NHE3 as indicated in A. B, summary of effect of agonists on transfected NHE3 activities. C, summary of effect of agonists on native basolateral Xenopus NHE (XNHE) activity in untransfected (A6/C1) and transfected A6 cell lines.
Regulation of NHE3 by Adenosine Is Mediated by CHP

Bail. mouse NHE3
Prey. CHP
G-gal activity

| 381 | 539 | +++ |
| 381 | 704 | ++ |
| 381 | 557 | ++ |
| 381 | 501 | +++ |

Fig. 6. C-terminal murine NHE3 bait used in two-hybrid system screens. Various constructs derived from the C terminus of murine NHE3 were used as baits against full-length CHP and prey. Strength of interaction was determined semi-quantitatively by β-galactosidase assay and is indicated by “++” (+ +, strong; + +, moderate; 0, no binding).

again in yeast. Insertion sizes were checked by BglII digestion. After direct sequencing, identical sequences were grouped via ClustalW at Pôle Bio-Informatique (Lyonnais) or via Pileup from Genetics Computer Group (Oxford) and overlaps connected by the Contig assembly program (Baylor College of Medicine, Houston, TX). Searches for protein relationships were performed using BLAST (NCBI, Bethesda, MD) (49). A number of positive prey were identified as either partial or full-length CHP. Truncated NHE3 baits were constructed as described above and tested against CHP. Brieﬂy, overnight cultures of yeast previously transformed with selected bait vectors were washed and transformed using a reaction mixture of small-scale yeast transformation media screens.

Immunoprecipitation and Immunoblot—Untransformed and transiently transfected OK cells, treated with either vehicle or CPA, were lysed in buffer (in mM: 150 NaCl, 50 Tris-HCL, pH 8, 5 EDTA, 1 EGTA, 1% (v/v) Triton X-100), and mixtures of protease inhibitors (in mg/ml) 10 phenylmethylsulfonyl fluoride, 4 leupeptin, 4 aprotinin, 10 pepstatin, and a mixture of protease inhibitors (in mg/ml) 57 M CPA (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA).

Confocal Fluorescent Imaging—OK cells were plated on glass coverslips, and A6 cells were plated onto permeant support filters (4.7 cm², Transwell; Costar, Cambridge, MA) previously coated with a thin layer of rat collagen. Transiently transfected cells (48 h later) were ﬁxed (4% formalin in PBS for 10 min), permeabilized (0.1% Triton X-100 in PBS for 10 min), blocked by 5% bovine serum albumin in PBS at 37 °C for 30 min. Samples were incubated with rabbit anti-NA polyclonal antibodies (1:200 dilution, Sigma) or mouse anti-FLAG monoclonal antibodies (1:200 dilution; Sigma) at 37 °C for 1 h, followed by a 6-hour wash with 0.1% Tween 20 in PBS, and immunofluorescence images were acquired and the pattern of expression of transporters was compared with that before treatment.

Expression and Preparation of siRNA Duplexes from the Target CHP Sense—Target region was selected from cDNA sequence of endogenous opossum CHP. By homology cloning we identiﬁed 300 nucleotides downstream of the CHP start codon (GenBank™ accession number: AY281349). The AA-N19 mRNA target was synthesized by Dharmacon Research Co. (Lafayette, CO), and sequences were as follows: 5'-aggagaacagaaag-3' (sense strand) and 5'-cuaucacauucgctu-3' (antisense strand). GL2 (ﬁreﬁsh luciferase gene) siRNA was synthesized according to Elbashir et al. (50). The 21-nucleotide siRNA duplex targeting OK mRNA sequence of the protein tyrosine kinase, Pyk2 (5'-aagccccguuagaa-gu-3') was kindly provided by Dr. Patricia Preisig (University of Texas Southwestern Medical Center, Dallas, TX). Oligonucleotides were provided at the concentration of 2 μM, puriﬁed, annealed duplex, and were ready to use for transfection. Approximately 24 h prior to transfection OK cells were plated at an appropriate cell density so that they were ~70% confluent the following day. For the complex formation, we used 7 μl of the TransIT-TKO transfection reagent (Mirus Corp., Madison, WI) in 100 μl of serum-free medium. Diluted TransIT-TKO reagent was incubated at room temperature for 20 min. Then siRNA (70 nM) was added to TransIT-TKO and incubated at room temperature for an additional 20 min. The TransIT-TKO reagent–siRNA complex mixture was given to OK cells, and cells were incubated with the mixture for 48 h. Assays for silencing were performed after 48 h of serum deprivation.

Statistical Analyses—Results are represented as means ± S.E. Quantitative differences between control and test conditions were assessed statistically by analysis of variance. Probability (p) of < 0.05 was considered statistically signiﬁcant.

RESULTS

Activation of A1R Inhibits NHE3 Activity—We previously showed that acute activation of A1R decreases NHE3 activity in renal epithelial cells (7, 9). This study uses OK cells expressing endogenous NHE3 and A6 cells expressing transiently transfected NHE3 to analyze the mechanism underlying A1R-induced inactivation of NHE3. As shown in Fig. 1A, activation of A1R in OK cells with 10⁻⁶ M CPA (N⁶-cyclopentyladenosine) reduced the activity of the native apical NHE3 by 31 and 35% at 15 and 30 min, respectively. As shown in Fig. 1 (B and C), CPA had similar effects on opossum or rat NHE3 stably transfected into A6 cells. A1R activation in the apical membrane by 10⁻⁶ M CPA for 15 or 30 min decreased the activity of transfected OK-NHE3 by 27 and 40%, respectively (Fig. 1B), and reduced the activity of transfected rat-NHE3 by 31 and 39%, respectively (Fig. 1C).

In both transfected and untransfected A6 cells, exposure to CPA (10⁻⁶ M for 15 min) inhibited the native basolateral Xenopus NHE (XNH) activity (Fig. 1D).

CPA Increases Surface NHE3 Protein—One mechanism that has been shown to acutely alter NHE3 activity is the change in
Regulation of NHE3 by Adenosine Is Mediated by CHP

from OK cells were immunoprecipitated (co-immunoprecipitation of native NHE3 and CHP. Lysates obtained (see Fig. 2A and Fig. 3A). In addition to biochemical biotin accessibility assays, we also studied CPA-induced changes of NHE3 distribution in live cells transiently expressing OK-NHE3 tagged with enhanced green fluorescent protein (OK-NHE3/eGFP). Fluorescent microscopy showed that CPA caused a visible decrease in surface NHE3 at 30 min of exposure to 10^{-6} M CPA (Fig. 2C). Comparable findings were obtained in A6 cells transiently expressing OK-NHE3/eGFP (Fig. 3C). Exposure to vehicle did not cause any change in NHE3/eGFP distribution in either cell lines (not shown).

Identification of Structural Elements Required for Down-regulation of NHE3 by CPA—According to the preceding results, inhibition of NHE3 activity by CPA at 15 min is exclusively due to a reduction of intrinsic NHE3 activity. We recently have shown that A1R-mediated inactivation of NHE3 is dependent on protein kinase C (PKC) (7, 9). Phosphorylation of NHE3 is an early event in regulation of NHE3 by protein kinases (36, 37, 55, 56). Elimination of phosphoserines on NHE3 may disrupt CPA regulation of NHE3 activity. To test this hypothesis, we assayed A6 cells stably expressing rat-NHE3 bearing either single or multiple serine substitutions for their response to CPA. As shown in Fig. 4A, substitution of serine residues at a single position (S605G) or multiple positions (S513G, S552A, S575A, S634A, S661A, and S690G) on NHE3 did not appreciably affect NHE3 inactivation by 10^{-6} M CPA. Similar results were obtained in studies using the PKC agonist PMA (phorbol 12-myristate 13-acetate). This is in contradistinction to the previous data in fibroblasts where phosphoserines were necessary but not sufficient for alteration of NHE3 activity by PMA (37). Co-exposure to the PKC antagonist calphostin C (10^{-8} M for 15 min) completely reversed NHE3 inhibition induced by either CPA or PMA (data not shown). CPA or PMA reduced the activity of basolateral XNHE in transfected cell lines to an extent that is similar to the effect of either of the agonist on XNHE in untransfected A6 cells (Fig. 4B). These findings suggest that direct phosphorylation of anti-NHE3 antibodies and immunoblotted (IB) with anti-CHP antibody. OK cells were treated with either vehicle or CPA (10^{-6} M, 15 min). B, in the left panel, OK cells were transfected with HA-tagged CHP (HA/CHP) and His6-tagged NHE3 (His/H3). Untransfected cells served as negative controls. The arrows indicate expected mobilities of NHE3 and CHP. The right panel shows co-immunoprecipitation of His/NHE3 with HA/CHP in OK cells treated with either vehicle or CPA (10^{-6} M, 15 min). C, co-immunoprecipitation of myc-tagged NHE3^655-662 (myc/NHE3^655-662) with HA/CHP in transfected and transected cells. Transfected cells were treated with either vehicle or CPA (10^{-6} M, 15 min). D: The left panel shows immunoprecipitation of myc-tagged NHE3^462 (myc/NHE3^462), and the right panel shows co-immunoprecipitation of myc-tagged NHE3^462 with HA/CHP (HA/CHP) in transfected and transected cells. Transfected cells were treated with either vehicle and the numbers in parentheses refer to the number of experiments performed under identical experimental conditions. The asterisk indicates the statistical difference compared with vehicle (*, p < 0.05, ANOVA).

Fig. 7. Co-immunoprecipitation of CHP-NHE3 in OK cells. A, co-immunoprecipitation of native NHE3 and CHP. Lysates obtained from OK cells were immunoprecipitated (IP) with either anti-CHP or cell surface NHE3 protein abundance (8, 40, 51–54). We used surface biotinylation to determine whether the decrease in NHE3 activity in response to activated A1R is associated with reduction in surface NHE3 antigen. As shown in Fig. 2, there was no detectable change in surface total native OK-NHE3 antigen after 15 min of 10^{-6} M CPA. However, 30 min of 10^{-6} M CPA induced a 35% reduction in surface NHE3. Similar results were obtained from studies A6 cells expressing transfected OK-NHE3 protein (Fig. 3, A and B). In both cell lines, CPA did not affect the amount of total cellular NHE3 protein (see Fig. 2A and Fig. 3A). In addition to biochemical biotin accessibility assays, we also studied CPA-induced changes of NHE3 distribution in live cells transiently expressing OK-NHE3 tagged with enhanced green fluorescent protein (OK-NHE3/eGFP). Fluorescent microscopy showed that CPA caused a visible decrease in surface NHE3 at 30 min of exposure to 10^{-6} M CPA (Fig. 2C). Comparable findings were obtained in A6 cells transiently expressing OK-NHE3/eGFP (Fig. 3C). Exposure to vehicle did not cause any change in NHE3/eGFP distribution in either cell lines (not shown).

Identification of Structural Elements Required for Down-regulation of NHE3 by CPA—According to the preceding results, inhibition of NHE3 activity by CPA at 15 min is exclusively due to a reduction of intrinsic NHE3 activity. We recently have shown that A1R-mediated inactivation of NHE3 is dependent on protein kinase C (PKC) (7, 9). Phosphorylation of NHE3 is an early event in regulation of NHE3 by protein kinases (36, 37, 55, 56). Elimination of phosphoserines on NHE3 may disrupt CPA regulation of NHE3 activity. To test this hypothesis, we assayed A6 cells stably expressing rat-NHE3 bearing either single or multiple serine substitutions for their response to CPA. As shown in Fig. 4A, substitution of serine residues at a single position (S605G) or multiple positions (S513G, S552A, S575A, S634A, S661A, and S690G) on NHE3 did not appreciably affect NHE3 inactivation by 10^{-6} M CPA. Similar results were obtained in studies using the PKC agonist PMA (phorbol 12-myristate 13-acetate). This is in contradistinction to the previous data in fibroblasts where phosphoserines were necessary but not sufficient for alteration of NHE3 activity by PMA (37). Co-exposure to the PKC antagonist calphostin C (10^{-8} M for 15 min) completely reversed NHE3 inhibition induced by either CPA or PMA (data not shown). CPA or PMA reduced the activity of basolateral XNHE in transfected cell lines to an extent that is similar to the effect of either of the agonist on XNHE in untransfected A6 cells (Fig. 4B). These findings suggest that direct phosphorylation of anti-NHE3 antibodies and immunoblotted (IB) with anti-CHP antibody. OK cells were treated with either vehicle or CPA (10^{-6} M, 15 min). B, in the left panel, OK cells were transfected with HA-tagged CHP (HA/CHP) and His6-tagged NHE3 (His/H3). Untransfected cells served as negative controls. The arrows indicate expected mobilities of NHE3 and CHP. The right panel shows co-immunoprecipitation of His/NHE3 with HA/CHP in OK cells treated with either vehicle or CPA (10^{-6} M, 15 min). C, co-immunoprecipitation of myc-tagged NHE3^655-662 (myc/NHE3^655-662) with HA/CHP (HA/CHP) in transfected and transected cells. Transfected cells were treated with either vehicle or CPA (10^{-6} M, 15 min). D: The left panel shows immunoprecipitation of myc-tagged NHE3^462 (myc/NHE3^462), and the right panel shows co-immunoprecipitation of myc-tagged NHE3^462 with HA/CHP (HA/CHP) in transfected and transected cells. Transfected cells were treated with either vehicle and the numbers in parentheses refer to the number of experiments performed under identical experimental conditions. The asterisk indicates the statistical difference compared with vehicle (*, p < 0.05, ANOVA).
NHE3 is not necessary for A1R-induced down-regulation of NHE3.

In the following series of experiments we tested the functional behavior of truncated versions of OK-NHE3 to identify the region in the NHE3 C terminus that is essential for the A1R response. Fig. 5 gives an overview on the truncated NHE3s screened for the ability of A1R activation to regulate NHE3. Compared with wild-type NHE3, mutants lacking the carboxyl tail distal to position 462 (NHE3<sub>462</sub>) were completely unresponsive to 15-min treatment with 10<sup>-6</sup> M CPA, whereas NHE3 truncated at position 552 (NHE3<sub>552</sub>) retained full response to CPA (Fig. 5B). Similarly, NHE3<sub>462</sub> lost PMA responsiveness.

**Fig. 8.** Subcellular localization/co-localization of CHP and NHE3 in OK and A6 cells. Constructs of HA/CHP and NHE3/eGFP were transfected either alone or in combination into OK or A6 cells. Forty-eight hours after transfection, cells were fixed and immunostained with HA antibody (Rhodamine Red-X conjugated) and observed by confocal microscopy. XZ and XY indicate orthogonal optical sections. The apical membrane is specified by an arrow on the XZ images. A, subcellular distribution of CHP and NHE3 in OK or A6 cells. B, co-localization of HA/CHP (red) and NHE3/eGFP (green) in OK cells. Co-localization is indicated by the merged yellow color. C, co-localization of HA/CHP and NHE3/eGFP in A6 cells. Color scheme is the same as in B.
whereas NHE3<sup>462–552</sup> and NHE3<sup>456–460</sup> were inhibited by 10<sup>-7</sup> M PMA (Fig. 5B). Co-exposure to calphostin C (10<sup>-8</sup> M for 15 min) prevented the response of OK-NHE3<sup>4552</sup> to either CPA or PMA (data not shown). The endogenous XNHE responded to either CPA or PMA in A6 cell lines expressing wild-type or truncated versions of OK-NHE3 (Fig. 5C). Various truncations of NHE3 per se did not significantly affect whole cell NHE3 protein expression (data not shown). We conclude that A<sub>1</sub>R-induced inhibition of NHE3 requires regulatory elements within residues 462–552 of the C-terminal domain of NHE3.

Identification of CHP as a CPA-regulated NHE3-binding Protein—By analogy to the molecular mechanism allowing inactivation of NHE3 by PKA, elevated Ca<sup>2+</sup>, or PKC (53, 57–60), we hypothesized that PKC-mediated inactivation of NHE3 by activated A<sub>1</sub>R involves interaction of NHE3 with a regulatory protein. In a yeast-two-hybrid screen of an adult mouse kidney cDNA library using full-length murine-NHE3 C terminus as bait, full-length murine CHP (calcineurin B homologous protein) was repeatedly positive in interacting clones. CHP has previously been reported to bind to NHE3 near the vicinity of the region of aa 462–552 essential for A<sub>1</sub>R-induced regulation (28). We next examined whether CHP interacts with the subdomain of NHE3 that is critical for A<sub>1</sub>R-dependent control of NHE3 activity. To this end, we used truncated versions of the C-terminal domain of murine NHE3 as bait in the yeast two-hybrid assay. As shown in Fig. 6, CHP specifically interacted with murine NHE3 aa 381–557, which is homologous to the CPA-sensitive domain on OK-NHE3 (aa 462–552). CHP interacted with a minimal version of the juxtamembrane C-terminal cytoplasmic domain of NHE3 comprising aa (murine) 338–501 (Fig. 6). In contrast, baits devoid of this region of the NHE3 C terminus showed no interaction with CHP (Fig. 6).

To test for association of CHP with full-length NHE3 in vertebrate cells and to determine whether CPA modulates the association, we performed co-immunoprecipitation studies. Immunoprecipitation of native NHE3 (Fig. 7A) brought down native CHP in the immune complex (Fig. 7A). Importantly, exposure to CPA increased the amount of NHE3 bound to native CHP by ~30% (Fig. 7, A and D). Additional evidence for an association of CHP and NHE3 was obtained in studies using OK transiently expressing His<sub>6</sub>-tagged rat-NHE3 and HA-tagged murine-CHP. As shown in Fig. 7B, anti-HA antibody co-precipitated His<sub>6</sub>-tagged rat-NHE3 only in double-transfected OK cells but not in OK cells that were not transfected (Fig. 7B) or solely transfected with His<sub>6</sub>-tagged rat-NHE3 (data not shown). Again, exposure of double-transfected OK cells to CPA increased the amount of NHE3 bound to CHP by 60% (Fig. 7, B and D).

If the critical interacting region on NHE3 is indeed aa 462–552, the association between CHP and NHE3 should be preserved with truncation of OK-NHE3 at aa 552 and disrupted by truncation of OK-NHE3 at aa 462. Co-transfection of myc-tagged OK-NHE3<sup>4552</sup> and HA-tagged CHP in OK cells showed intact co-immunoprecipitation as well as increased NHE3-CHP association in response of CPA (Fig. 7C), whereas co-expression of myc-tagged OK-NHE3<sup>462</sup> and HA-tagged CHP in OK cells demonstrated no association of NHE3 with CHP (Fig. 7D). Fig. 7E summarized the CPA-mediated increases of association of CHP with NHE3 (native and transfected proteins).

Subcellular Localization of CHP and NHE3 in OK Cells and A6 Cells—In the next series of experiments we compared subcellular localization of NHE3 and CHP in OK cells and A6 cells transiently expressing HA-tagged CHP (HA/CHP) and NHE3/eGFP by confocal fluorescent microscopy. HA-tagged CHP showed primarily intracellular distribution (Fig. 8A, upper two panels, xx section), whereas NHE3/eGFP localizes to both intracellular compartment as well as the apical membrane (Fig. 8A, bottom two panels, xx section). However, there is definite co-localization of HA/CHP and NHE3/eGFP in the apical membrane in both OK and A6 cells (Fig. 8, B and C, yellow signal of the merged composite).

Functional Importance of CHP-NHE3 Interaction for CPA-dependent Control of NHE3 Activity—We used two approaches to define the functional significance of the CPA-NHE3 interaction in A<sub>1</sub>R-dependent control of NHE3 activity. First we attempted to create a “dominant negative” NHE3 polypeptide. We transiently transfected a construct expressing aa 462–552 of OK-NHE3 (NHE3<sup>462–552</sup>) in OK cells to attempt to stoichiometrically bind endogenous CHP and competitively inhibit the endogenous CHP-NHE3 interaction. To validate this approach, we first established that transfected NHE3<sup>462–552</sup> actually competes with wild-type full-length NHE3 for CHP binding. To this end, OK cells were triple-transfected with HA-tagged CHP (HA/CHP), His<sub>6</sub>-tagged NHE3 (NHE3/6H), and FLAG-tagged NHE3<sup>462–552</sup> (FLAG-NHE3<sup>462–552</sup>). Lysate from vehicle or CPA-treated cells were subjected to immunoprecipitation by anti-His<sub>6</sub> or anti-FLAG antibodies and immunoblotted with anti-HA antibody. As illustrated in Fig. 9, both NHE3<sup>462–552</sup> and full-length NHE3 interacts with CHP. Interestingly, in the presence of NHE3<sup>462–552</sup>, CPA failed to increase binding of
full-length NHE3 to CHP (Fig. 9). To further substantiate that NHE3 and NHE3\(^{462-552}\) compete for CHP binding, we examined OK cells transiently expressing HA/CHP, NHE3/eGFP, and FLAG/NHE3\(^{462-552}\). Forty-eight hours after transfection, cells were fixed and immunostained with specific antibodies. To FLAG/NHE3\(^{462-552}\), images a pseudo-color of either blue (A) or green (B, right) was assigned. Double staining was performed using HA and FLAG antibodies. XZ and X:Y indicate orthogonal optical sections. The apical membrane is specified by an arrow on the XZ images. A, subcellular distribution of CHP, NHE3 and NHE3\(^{462-552}\). B, co-localization of CHP with NHE3 (left image) or NHE3\(^{462-552}\) (right image).

Fig. 10. Localization of NHE3\(^{462-552}\) in OK cells: relative co-localization with NHE3 and CHP. OK cells were triple transfected with HA/CHP, NHE3/eGFP, and FLAG/NHE3\(^{462-552}\). Forty-eight hours after transfection, cells were fixed and immunostained with specific antibodies. To FLAG/NHE3\(^{462-552}\), images a pseudo-color of either blue (A) or green (B, right) was assigned. Double staining was performed using HA and FLAG antibodies. XZ and X:Y indicate orthogonal optical sections. The apical membrane is specified by an arrow on the XZ images. A, subcellular distribution of CHP, NHE3 and NHE3\(^{462-552}\). B, co-localization of CHP with NHE3 (left image) or NHE3\(^{462-552}\) (right image).

**DISCUSSION**

In addition to maintenance of sodium homeostasis under physiological conditions, a rise in adenosine after renal ischaemia has protective effects against renal cell damage via a multitude of intrarenal and extrarenal mechanisms (2, 61–63). Regulation of NHE3 activity by adenosine may represent a versatile mechanism to adapt the energy supply/demand balance of the proximal tubule under physiological as well as certain pathophysiological conditions. Our recent findings support a role of A\(_1\)R activation in modulation of proximal tubule Na\(^+\) transport via regulation of NHE3 (7, 9). Using homologous as well as heterologous systems, we found that acute A\(_1\)R
Fig. 12. Influence of knockdown of endogenous CHP on NHE3 total protein/activity and CPA-induced inhibition of NHE3 activity in OK cells. A, amino acid sequence alignment of the cloned opossum CHP with the C terminus of murine, rat, and human CHP1 sequence. B, representative immunoblot and quantification of CHP total protein content in untransfected and CHP, Pky2, or GL2 siRNA-transfected OK cells. D, regulation of NHE3 by Adenosine Is Mediated by CHP.
stimulation inhibits NHE3 primarily via a Ca\(^{2+}\)-sensitive, PKC-coupled pathway (7, 9). In the present study we report that stimulation of \(\alpha_1\)R affects NHE3 in a mode similar to that of parathyroid hormone, dopamine, or \(\alpha_1\)-R activation (8, 40, 51, 52). Early effects (\(<\)30 min) of \(\alpha_1\)R regulate intrinsic transport activity of NHE3, and more sustained (\(>\)30 min) inhibition is associated with reduction of NHE3 protein in the plasma membrane. Reduction of NHE3 cell surface expression (within 30 min) was not accompanied by a comparable decreased of NHE3 activity at 30 min. The present findings are quite similar to that seen in response to parathyroid hormone or dopamine in OK cells (40, 52). The molecular mechanisms underlying this observation are currently unknown. Two hypothetical models have been proposed that may explain the findings (52). In the first model, at 15 min only a fraction of surface NHE3 is down-regulated. This same fraction at 30 min undergoes endocytosis leaving the remaining surface transporters running at a rate similar to control conditions. In the second model, all surface NHE3 is inhibited (within 15 min), but only a restricted population of NHE3 becomes internalized at 30 min. Those transporters that are not targeted to be internalized recover their transport activity with time (30 min). Currently, there are no data to discern these two paradigms.

In fibroblasts, phosphorylation of NHE3 may have a permissive role in regulation of NHE3 activity by PKC (37), but NHE3 phosphorylation is insufficient to mediate the functional regulation (37, 64). In A6 cells, rat NHE3 bearing either single or six substitutions of PKC phosphorylation sites retained functional regulation of transport activity by \(\alpha_1\)R activation. However, our present findings do not exclude the possibility that other phosphorylated sites on the NHE3 C terminus are involved in \(\alpha_1\)-R-induced inactivation of NHE3. \(\alpha_1\)-R-induced NHE3 inhibition requires the integrity of a domain located between amino acids 462 and 552 of the opossum NHE3 sequence. This domain does not include classic PKC consensus sites compatible with the notion that activation of \(\alpha_1\)R does not involve changes in NHE3 phosphorylation. A cofactor recently identified to bind in the vicinity of this region is CHP (28). By screening for proteins that may function as regulators of NHE3 in a yeast two-hybrid study, we provided evidence that CHP interacts directly with a region of NHE3 corresponding to the domain that mediates \(\alpha_1\)-R-induced inhibition. The juxtamembrane localization of the binding domain of CHP on NHE3 is consistent with a previous report (28). This prompted us to study the role of CHP in NHE3 regulation by adenosine.

Association of CHP with NHE3 in vivo was confirmed by co-immunoprecipitation in OK cells with either native or transfected NHE3 and CHP. OK cells have been shown recently to predominantly express the endogenous CHP2 isoform (65). However, our findings demonstrate that the OK cell CHP exhibits highest homology with human and murine CHP1 but not CHP2. There is considerable heterogeneity among different OK cells, and our cell line may express both CHP1 and CHP2 but the reverse transcription-PCR with CHP1-derived primers selectively picked out OK CHP1 rather than CHP2. Similar to reported data on direct interaction of cloned CHP from different species with NHE3 (28, 65), our results in OK cells indicate interaction of CHP with NHE3 under basal conditions. In addition, we showed that NHE3-CHP binding increases upon \(\alpha_1\)-R stimulation with CPA, and we further provided functional data to support the role of this interaction in regulation of NHE3 by either CHP.

The CHP-interacting region on NHE3 derived from the yeast two-hybrid data was confirmed in OK cells by the observation that truncation of NHE3 at aa 552 still retained baseline and CPA-activated association of CHP with NHE3. In contrast, truncation of NHE3 at aa 462 showed no binding of NHE3 to CHP. The functional significance of this interaction was supported by additional findings. Truncation of NHE3 beyond aa 552 abolished CPA-induced regulation. This finding per se does not rule out regulatory functions other than CHP binding in this region. However, transient overexpression of an NHE3 peptide, as 462–552, which competes for binding of CHP to NHE3, prevented CPA-induced early inhibition of NHE3 activity and CPA-mediated binding of CHP to NHE3. The functional role of CHP was further secured by the fact that reduction of native CHP by siRNA abolished the ability of CPA to inhibit NHE3 activity. Importantly, reduction of native CHP by siRNA influenced baseline NHE3 activity and total NHE3 protein abundance suggesting that CHP may influence NHE3 regulation by activating diverse mechanisms.

As noted above, \(\alpha_1\)-R activation inhibits NHE3 by decreasing its intrinsic activity as well as surface protein. The mechanism whereby CHP binding to aa 462–552 affects intrinsic transport NHE3 activity is unknown. It has recently been suggested that Ca\(^{2+}\) allows a conformational change of CHP and that conformational changes in CHP transduces cellular Ca\(^{2+}\) signals to other cellular proteins, somewhat akin to the role of calmodulin (66). Furthermore, CHP has potential phosphorylation sites for PKC (26). It is conceivable that a finite number of NHE3 proteins are bound to CHP under basal conditions. After CPA stimulation, the Ca\(^{2+}\)-induced conformational change in CHP stimulates further association of CHP to NHE3, although Ca\(^{2+}\)-dependent binding of CHP to NHE1 could not be demonstrated with recombinant proteins in vitro (28). PKC upon activation moves from the cytoplasm to plasmalemma. Because CHP1 associates with microtubules (30) and the microtubule surface may provide a scaffold of two or more factors that otherwise do not directly interact (67), it is possible that the microtubule surface is responsible for bringing PKC into the vicinity of NHE3. Phosphorylation of NHE3-bound CHP may then inactivate NHE3 by directly altering the transport subdomain of NHE3. It has been proposed that CHP attenuates the stimulation of NHE1 by serum and a mutationaly activated GTPase in CCL39 cells by a change of CHP phosphorylation state (26, 68).

Comparable to previous findings in PS120 fibroblasts and OK cells expressing exogenous CHP2 (28, 65), CHP partly co-localized with NHE3 in the surface membrane in OK cells and A6 cells. Microtubule dynamics appear to play a role in membrane trafficking events (69), and EF-hand Ca\(^{2+}\)-binding microtubule-interacting proteins, such as p22/CHP1, have

60 \(\mu\)g of total lysate was immunoblotted by anti-CHP antibody. Cells were exposed to transfection reagent either without siRNA (Untransfected) or with siRNA against CHP (CHP siRNA), protein tyrosine kinase (Ptyk2 siRNA) or non-mammalian protein (GL2 siRNA). The arrow indicates expected mobility of CHP. C, immunostaining of native CHP in untransfected (left image) and siRNA transfected OK cells (right image). Immunostaining was performed using anti-CHP antibody. XZ and XY indicate orthogonal optical sections. The apical membrane is indicated by an arrow on the XZ image. D, typical immunoblot and quantification of native CHP. NHE3, and Na/K ATPase total protein content in untransfected and CHP siRNA-transfected OK cells. 60 \(\mu\)g of total lysate was immunoblotted by anti-CHP, anti-NHE3 (#3688), or anti-Na/K ATPase (\(\alpha_2\)Z), respectively. E, NHE3 activity of untransfected or CHP siRNA-transfected OK cells in response to a 15-min treatment with 10\(^{-6}\) M CPA or vehicle. Data presented are means \pm S.E. The numbers in parentheses refer to the number of experiments performed under identical experimental conditions. The asterisk indicates the statistical difference compared with untreated cells (*, \(p < 0.05; **, p < 0.01\) ANOVA).
been suggested to influence membrane trafficking by effecting either directly the components of the vesicle transport machinery or indirectly the organization of the cytoskeleton (30). NHE3 is sensitive to the organization of the cytoskeleton (70). It is possible that CHP can exert its effect by altering the organization of the cytoskeleton. In the present study we also demonstrated that decreased surface expression of NHE3 is associated with inhibition by prolonged exposure of A1R to CPA. CHP1 is involved in targeting/fusion of transcytotic vesicles with the apical membrane (25). Although, NHE3 is endocyted into clathrin-coated vesicles (40, 71), exactly how CHP regulates NHE3 trafficking is unclear.

In summary, we present evidence for a novel function of the ubiquitous Ca2+-binding protein CHP. In epithelial cells of the kidney, we have shown that activation of A1R leads to increased interaction of NHE3 with CHP. Physical interaction of NHE3 with CHP is necessary for acute regulation of the intrins transport activity of NHE3.

Acknowledgments—We are grateful to Dr. Diane L. Barber (University of California, San Francisco, CA) for the anti-CHP antiserum and to Dr. Patricia Preisig (University of Texas Southwestern Medical Center, Dallas, TX) for the Pkoy2 siRNA. The monoclonal antibody a2F2 developed by Douglas M. Fambrough was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA.

REFERENCES

1. Poulsen, S. A., and Quinn, R. J. (1998) Bioorg. Med. Chem. 6, 619–641.
2. Jackson, E. K., and Dubey, R. K. (2001) Am. J. Physiol. 281, F597–F612.
3. Okusa, M. D. (2002) Am. J. Physiol. 282, F10–F18.
4. Spielman, W. S., and Arend, L. J. (1991) Hypertension 17, 117–130.
5. Kii, F., Sejoest, O. M., and Steen, P. A. (1986) Int. J. Biochem. 12, 245–250.
6. Ostensen, J., Stokke, E. S., Hartmann, A., Wensell, K., and Kiil, F. (1989) Acta Physiol. Scand. 137, 189–198.
7. Di Sole, F., Casavola, V., Mastrobattardino, L., Verrey, F., Moe, O. W., Burchardt, G., Murer, H., and Helme-Kolb, C. (1999) J. Physiol. 515, 829–842.
8. Di Sole, F., Cerulli, R., Casavola, V., Moe, O. W., Burchardt, G., and Helme-Kolb, C. (2002) J. Physiol. 541, 529–543.
9. Dinour, D., Agmon, Y., and Brezis, M. (1993) J. Biol. Chem. 268, 14993–14999.
10. Blond, D. M., and Whittam, R. (1964) Int. J. Biochem. 4, S3–S8.
11. Numata, M., Petrecca, K., Lake, N., and Orlowski, J. (1998) J. Biol. Chem. 273, 6951–6959.
12. Gundersen, G. G., and Cook, T. A. (1999) J. Cell Biol. 145, 1023–1034.
13. Kurihara, K., Yu, F. L., Zhu, C., and Tofovic, S. P. (2002) J. Biol. Chem. 277, 8421–8428.
14. Zoukhri, D., and Donowitz, M. (2003) Am. J. Physiol. Cell Physiol. 285, C1527–C1536.
15. Jackson, E. K., Zhu, C., and Tofovic, S. P. (2002) Am. J. Physiol. 283, F41–F51.
16. Welch, W. J. (2002) Curr. Opin. Pharmacol. 2, 165–170.
17. Oie, J., and Quinn, R. J. (1999) Drug Discov. Today 4, 542–551.
18. Yamakawa, S., Shiogawa, H., and Hiraoka, T. (2000) Biochem. Pharmacol. 60, 1499–1504.
19. Meyer, T., and York, J. D. (1999) Nat. Cell Biol. 2, 94–105.
20. Wiedeke, M., R., Zhao, H., and Moe, O. W. (1999) J. Biol. Chem. 274, 11289–11295.
21. Gierse, R., and Schiestl, R. H. (1999) Methods Mol. Cell Biol. 5, 255–269.
22. Mo, E. W. (1999) J. Clin. Invest. 104, 1141–1146.
23. Lin, X., and Barber, D. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12631–12636.
24. Lin, X., and Barber, D. L. (1999) J. Biol. Chem. 274, 36125–36131.
25. Pang, T., Su, X., Watabashy, S., and Shiogawa, M. (2001) J. Biol. Chem. 276, 17367–17372.
26. Matsuzaki, K., Miyake, Y., Nagita, M., Inoue, H., Shihtukbo, D., Takemoto, K., Ohsutaka, C., Murakami, H., Nakamura, N., and Kanazawa, H. (2001) J. Biochem. (Tokyo) 130, 217–225.
27. Timm, S., Titus, B., Bernd, K., and Barroso, M. (1999) Mol. Biol. Cell 10, 3473–3488.
28. Nakamura, N., Miyake, Y., Matsushita, M., Tanaka, S., Inoue, H., and Kanazawa, H. (2002) J. Biochem. (Tokyo) 131, 483–491.
29. Helme-Kolb, C., Montrose, M. H., Stange, G., and Murer, H. (1999) Pflugers Arch. 415, 461–470.
30. Verrey, F. (1994) J. Membr. Biol. 138, 65–76.
31. Casavola, V., Guerra, L., Reshkin, S. J., Jacobson, K. A., and Murer, H. (1997) Mol. Pharmacol. 51, 516–523.
32. Presto, A. S., Muller, J., and Handler, J. S. (1988) Am. J. Physiol. 255, C1527–C1536.
33. Wang, T., Hropot, M., Aronson, P. S., and Giebisch, G. (2001) Am. J. Physiol. 280, F1117–F1122.
34. Chou, J. Y., Shah, M., Lee, M. G., Schultheis, P. J., Shull, G. E., Mualem, S., and Baum, M. (2000) J. Clin. Invest. 105, 1141–1146.
35. Barroso, M. R., Bernd, K., DeWitt, N. D., Chang, A., Mills, K., and Suttal, E. S. (1996) J. Biol. Chem. 271, 10183–10187.
36. Lin, X., and Barber, D. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12631–12636.