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Protein mediated regulation of the NHE1 isoform of the Na^+/H^+ exchanger in renal cells. A regulatory role of Hsp90 and AKT kinase

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ABSTRACT

Na^+/H^+ exchanger isoform one (NHE1) is a pH regulatory protein that is present in renal tissues and serves to remove protons from within cells and protect against intracellular acidification. NHE1 has a large 315 amino acid cytosolic regulatory domain that regulates the catalytic membrane domain. We examined protein-mediated regulation of NHE1 through the cytosolic domain. Affinity chromatography with the C-terminus of NHE1 yielded a number of NHE1 binding proteins including 14-3-3 protein, heat shock proteins (Hsp90 and Hsp70) and Na^+/K^+ ATPase. We confirmed that 14-3-3 and heat shock proteins bind to or regulate NHE1 but could not confirm that Na^+/K^+ ATPase binds to the intact protein. The Hsp90 inhibitor 17-AAG decreased NHE1 activity and NHE1 phosphorylation in MDCK cells but did not decrease protein levels. Additionally, 17-AAG decreased phospho-AKT levels. Direct inhibition of AKT with MK2206 decreased NHE1 activity, though this effect was not additive with the effect of 17-AAG. The results demonstrate that in renal cells, NHE1 is associated with several regulatory proteins including Hsp90, and that Hsp90 affects its function possibly through altered phosphorylation of the protein *via* the AKT kinase.

1. Introduction

The Na^+/H^+ exchanger isoform-1 (NHE1) is a ubiquitously expressed plasma membrane glycoprotein which functions to protect cells from intracellular acidification by extruding one intracellular proton in exchange for a single extracellular sodium [1]. Ten isoforms of Na^+/H^+ exchanger have been discovered (NHE1-NHE10). NHE1 was the first isoform discovered and is ubiquitously distributed [1,2]. Other isoforms have more restricted tissue distributions and some have predominantly intracellular localization. In mammals, aside from its role in regulation of intracellular pH, NHE1 is also important in regulation of cell volume, cell proliferation, differentiation and in metastasis of some types of tumor cells [1,3,4].

NHE1 has two major domains. The N-terminal membrane domain is approximately 500 amino acids and transports ions. This is followed by a hydrophilic, carboxyl-terminal cytosolic domain that is 315 amino acids in human NHE1 [2]. The cytosolic domain regulates the membrane domain. Some of the regulation is by phosphorylation occurring in the distal 178 amino acids of the cytosolic domain [5,6]. The regulation of NHE1 shows tissue specificity and this may be a reflection of the presence of various receptors, different regulatory kinases, phosphatases or other regulatory proteins present in the various tissues in which NHE1 is found [7].

NHE1 is ubiquitous in mammalian tissues. One tissue in which it is present is the kidney. There it has been identified by western blotting, measurement of its activity, or identification of its mRNA. The different renal cell lines and tissues in which it has been identified include human embryonic kidney cells [8] renal tubules [9], in inner medullary collecting duct [10], in whole renal cortex [11], Madin-Darby canine kidney (MDCK) cells [12], rat proximal tubule cells [13], and in M-1 cortical collecting duct cells [14]. Reports show that it is present on both the apical and basolateral membrane of polarized renal cells MDCK cells [15] though it is usually regarded as being present on the basolateral membrane [16,17]. In the kidney, NHE1 is involved in NaCl absorption, $NaHCO_3$ absorption, pH regulation and maintenance of cell volume (reviewed in [17]). We recently demonstrated that NHE1 in the kidney is regulated by Erk-dependent phosphorylation of the regulatory cytosolic tail [18]. While this undoubtedly is an important part of NHE1 regulation, regulation of NHE1 is thought to be through a combination of phosphorylation of the distal tail, and through protein-protein mediated interactions which can occur in either more proximal or more distal regions of the regulatory tail [5,7]. Regulation of the NHE1 protein by protein-protein interactions has been documented in number of tissues where NHE1 has been shown to bind and be regulated by calmodulin [19], calcineurin homologous protein [20], B-Raf [21], heat shock proteins [22], carbonic anhydrase [23], phosphatases [24,25]

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and other proteins (reviewed in [7]).

In this study, we examine protein-mediated regulation of the NHE1 isoform of the Na^+/H^+ exchanger in renal cells. Using affinity chromatography and mass spectrometry, we identified a profile of proteins that bind to the cytoplasmic tail of NHE1 in the kidney. We confirmed the interaction of 14-3-3 and heat shock proteins (Hsps), Hsp70 and Hsp90 by co-immunoprecipitation and immunoblotting. Akt is a serine/threonine kinase involved in cell proliferation, migration and metabolism. We show, for the first time, that Hsp90 interaction with NHE1 may be involved in the Akt-mediated regulation of NHE1. Our results are the first demonstration of the mechanisms involved in protein-mediated regulation of this protein in the mammalian kidney.

2. Materials and methods

2.1. Materials

Mouse kidneys were purchased from Pel Freeze Biologicals, Rogers, AR, USA, Affi-Gel 10 was from Bio-Rad Laboratories, (Mississauga, Ont., Canada). Hsp70 and Hsp90 antibodies were from Enzo life sciences (Brockville, Ont., Canada), anti 14-3-3 antibody was from Santa Cruz Biotechnology (Dallas, Texas, USA), anti Na^+/K^+ ATPase antibody (α6f, anti-alpha 1 subunit) was from the Developmental Studies Hybridoma Bank (University of Iowa). Anti Polyclonal anti hemagglutinin (HA) antibody was from Santa Cruz, anti Hsp90 monoclonal antibody (AC88) was from Enzo Scientific. Anti-Akt protein antibody was from Cell Signaling Technology. 17-AAG (17-*N*-allylamino-17-demethoxygeldanamycin) was obtained from Cayman Chemical Co. Ann Arbor, Michigan, USA. MK-2206 was from Selleck Chemicals LLC. DSP (Dithiobis(succinimidylpropionate)) was purchased from Pierce, (Rockford, IL). Anti-NHE1 antibody was from BD Biosciences.

2.2. Protein production for affinity purification

To identify NHE1 interacting proteins we used affinity chromatography as described earlier [21]. We produced the C-terminal of rabbit NHE1, amino acids 545–816, as a fusion protein with glutathionine-S-transferase essentially as described earlier [22]. Induction was at 30 °C with isopropyl β-D-thiogalactopyranoside. Purification of the fusion protein was with glutathione sepharose affinity chromatography. As a control, glutathionine-S-transferase was produced and purified using the same plasmid without an insert.

Kidney extracts were prepared from 10 frozen mouse kidneys cut into appropriately sized pieces for homogenization. Kidneys were homogenized in a Bullet Blender (Next Advance Inc., USA) using equal volume 0.5 mm zirconium oxide beads and 2 volumes of homogenization buffer (20 mM HEPES, pH 7.6, 125 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithioreitol, 1 × Protease Inhibitor Cocktail (Roche Applied Science), 1 mM PMSF). The homogenate was centrifuged twice, first at 4000 rpm for 10 min and then at 35,000 rpm for 1 h at 4 °C in an ultracentrifuge. The supernatant was collected from the second spin and frozen prior to use in affinity chromatography.

Homogenization was with a Bullet Blender (Next Advance, HY, USA) according to the manufacturers protocol. Nuclei and debris were removed by centrifugation at 10,000 rpm × 10 min. The supernatants were then pooled and centrifuged twice at 4000 rpm × 10 min at 4 °C. The next supernatant was centrifuged for one hour at 35,000 rpm for one hour at 4 °C. The final supernatant was collected and frozen prior to use in affinity chromatography.

For affinity chromatography, 5 mg of purified GST or 2.5 mg of purified GST-NHE1 fusion protein (amino acids 545–816) were coupled to Affi-Gel 10 as described by the manufacturer. After blockage of any remaining reactive esters and washing, the resin was prepared by washing in buffer A. The columns were incubated with 5 ml of kidney extract at 4 °C overnight with gentle agitation. Resins were drained and washed with 50 volumes of Buffer A containing 500 mM NaCl, 0.5% Nonidet P-40 and 1 mM PMSF followed by 50 volumes of Buffer A with 1 mM PMSF. Proteins bound to the resins were eluted with Buffer A containing 1% SDS at room temperature. Eluted proteins were precipitated with trichloroacetic acid, the pH was neutralized and the sample was run on SDS-PAGE.

2.3. Mass spectrometry

Proteins eluted from either control (GST) affinity chromatography or experimental (NHE1-GST) were analyzed by mass spectrometry. In-gel trypsin digestion was performed on samples that were run on SDS-PAGE. Briefly, the excised gel bands were destained twice in 100 mM ammonium bicarbonate/acetonitrile (50:50). The samples were then reduced (10 mm BME in 100 mm bicarbonate) and alkylated (55 mM iodoacetamide in 100 mM bicarbonate). After dehydration enough trypsin (6 ng/μl) was added to just cover the gel pieces and the digestion proceeded overnight (~16 h.) at room temperature. Tryptic peptides were initially extracted from the gel using 97% water/2% acetonitrile/1% formic acid. This was followed by a second extraction using 50% of the first extraction buffer and 50% acetonitrile.

Fractions containing tryptic peptides were dissolved in aqueous 25% *v*/v acetnonitrile and 1% *v*/v formic acid were resolved and ionized by using nanoflow HPLC (Easy-nLC II, Thermo Scientific) coupled to the LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Scientific). Nanoflow chromatography and electrospray ionization were accomplished by using a PicoFrit fused silica capillary column (ProteoPepII, C18) with 100 μm inner diameter (300 Å, 5 μm, New Objective). Peptide mixtures were injected onto the column at a flow rate of 3000 nL/min and resolved at 500 nL/min using 45 min linear gradients from 0 to 45% *v*/v aqueous ACN in 0.2% *v*/v formic acid. The mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey Orbitrap spectra using external mass calibration, with a resolution of 60, 000 and *m/z* range of 400–2000. The fourteen most intense multiply charged ions were sequentially fragmented by using collision induced dissociation, and spectra of their fragments were recorded in the linear ion trap; after two fragmentations all precursors selected for dissociation were dynamically excluded for 60 s. Data was processed using Proteome Discoverer 1.4 (Thermo Scientific) and the Uniprot mouse database was searched using SEQUEST (Thermo Scientific). Search parameters included a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.8 Da. Peptides were searched with carbamidomethyl cysteine as a static modification and oxidized methionine and deamidated glutamine and asparagine as dynamic modifications.

2.4. Cell culture and stable transfection

MDCK cells were maintained essentially as described earlier [18]. They were passaged in Ham's F12/Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum and 50 μg/ml Gentamicin. Cells were maintained at 37 °C in 5% CO_2. Where indicated, MDCK cells were stably transfected with the plasmid pYN4 + which contains the full-length human NHE1 protein with a hemagglutinin tag [26]. Lipofectamine™ 2000 reagent was used for transfection in accordance with the manufacturer's instructions. Transfected cells were selected with 800 μg/ml Geneticin (G418).

2.5. Immunoprecipitation of phosphorylated NHE1

For some experiments MDCK cells expressing HA tagged NHE1 were labeled with [^{32}P] inorganic phosphate. The standard media was removed and plates were washed 2 times with phosphate-free DMEM. Cells were then incubated in phosphate-free DMEM (8 ml) for 30 min at 37 °C and were then were washed 2 × with phosphate-free DMEM. Phosphate-free DMEM (2 ml) was added with $H_3{}^{32}PO_4$ at a final concentration of 100 μCi/ml media. Cells were incubated with [^{32}P]

for 3 h. Cell lysates were then used for immunoprecipitation of NHE1 as described below.

2.6. Intracellular pH measurement

MDCK cells were grown on coverslips and were incubated overnight in reduced (0.5%) serum media. BCECF was used to measure the rate of intracellular pH (pH*i*) recovery after acute acid load by ammonium chloride (50 mM × 3 min. followed by withdrawal). NHE1 activity was measured as the slope of the first 20 s of recovery from acidification and was expressed as ΔpH/sec as described earlier [18,27]. Where indicated, cells were subjected to two consecutive ammonium chloride pulses and were monitored using a PTI Deltascan spectrofluorometer as previously earlier [28]. NH_4Cl prepulse was used to acidify the cells both times. In the first pulse, acidification of cells by NH_4Cl removal was followed by ~20 s incubation in a Na^+-free buffer, after which the cells were allowed to recover in a normal Na^+ buffer. The second pulse was the same except that acidification induced by NH_4Cl withdrawal was allowed to occur for 3 min in Na^+-free buffer prior to recovery in normal Na^+ buffer.

In some experiments cells were subjected to sustained intracellular acidosis essentially as described earlier [18,28]. Sustained intracellular acidosis was induced by treating cells with ammonium chloride, followed by withdrawal and maintaining the cells in sodium free medium for 3 min as described earlier.

2.7. Immunoprecipitations

To determine if NHE1 and other proteins interact *in vivo*, we used co-immunoprecipitation essentially as described earlier [23]. We chose four proteins to examine, based on examination of the proteins that interacted with the regulatory cytosolic tail of the NHE1 (Table 1). Immunoprecipitations were from MDCK cells that had been stably transfected with the plasmid pYN4 + to express the entire cDNA for the coding region of the Na^+/H^+ exchanger with a HA tag [29]. For immunoprecipitations, cells (100 mm dishes) were washed with phosphate buffered saline (PBS, 150 mM NaCl, 5 mM sodium phosphate, pH 7.4) and then incubated on ice for 10 min in 500 μl of RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 80 mM NaF, 5 mM EDTA, 1 mM EGTA, 1 mM Na-orthovanadate, 1% NP-40, 0.05% deoxycholate and protease inhibitor cocktail). The cells were collected and centrifuged for 10 min @ 10,000*g* at 4 °C. The cell lysate was pre-cleared with agarose at 4 °C for 30 min followed by centrifugation at 4 °C for 30 s. The supernatant was incubated with goat polyclonal anti-HA agarose-conjugate antibody (Santa Cruz) with gentle agitation overnight at 4 °C. Beads were collected by centrifugation at 3000 rpm × 30 s and washed 3 × with PBS. After washing, the bound protein was eluted from the washed beads by incubating with 40 μl of 2 × SDS-PAGE sample loading buffer at 37 °C for 15 min and immunoblotted as described below. In some experiments, where indicated, cells were treated with DSP to obtain a more quantitative co-immunoprecipitation. DSP was added to cells at a final concentration of 2 mM for 30 min at room temperature. The reaction was terminated by addition of Tris, pH 7.5, to a final concentration of 10 mM. Cells were then washed with phosphate-buffered saline, and the immunoprecipitation was continued as described above. In some experiments cells were treated with 10 μM MK-2206 for 18 h prior to immunoprecipitations.

2.8. Statistics

All data are expressed as means ± SE and plotted with KaleidaGraph 4.1 (Synergy Software, PA, US). For intracellular pH measurements, the reported results are those of 10 experiments per treatment/condition. The results of western blot analyses for MAPK assays and treatments with 17-AAG and MK2206; and *in vivo* phosphorylation assays, are those of a minimum of three experiments per treatment/condition. Statistics for all experiments were calculated using Wilcoxon Mann-Whitney rank sum test. A P-value < 0.05 was

Table 1
List of NHE1-interacting peptides unique to PCRB-GST *versus* GST-control analysis. PSM, peptide spectral match.

Accession #	Protein	PSM	Function
P63017	Heat Shock cognate 71 kDa protein	68	Signal transduction
A2ARV4	Low-density lipoprotein receptor-related protein 2	28	Molecular chaperone
Q9D0K2	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial	11	Metabolism
Q8VDN2	Sodium/potassium-transporting ATPase subunit α-1	10	Ion transport
Q61696	Heat shock 70 kDa protein 1A	8	Signal Transduction
P60710	Actin, cytoplasmic 1	7	Cell motility
P17751	Triosephosphate isomerase	7	Metabolism
P16858	Glyceraldehyde-3-phosphate dehydrogenase	6	Metabolism
P16125	L-lactate dehydrogenase B chain	6	Metabolism
O88338	Cadherin-16	5	Cell adhesion
P26443	Glutamate dehydrogenase 1, mitochondrial	5	Metabolism
P11499	Heat shock protein HSP 90-β	5	Signal transduction
P63101	14-3-3 protein zeta/delta	4	Signal transduction
Q9CQV8	14-3-3 protein β/α	3	Signal transduction
Q99KI0	Aconitate hydratase, mitochondrial	3	Metabolism
Q9CPY7	Cytosol aminopeptidase	3	Housekeeping
Q61425	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	3	Metabolism
P14152	Malate dehydrogenase, cytoplasmic	3	Metabolism
P09405	Nucleolin	3	Biosynthesis
Q9D051	Pyruvate dehydrogenase E1 component subunit beta	3	Metabolism
Q99L13	3-hydroxyisobutyrate dehydrogenase, mitochondrial	2	Metabolism
Q8VCR7	α/β hydrolase domain-containing protein 14B	2	Transcription
Q8K0L3	Acyl-coenzyme A synthetase ACSM2, mitochondrial	2	Metabolism
P45376	Aldose reductase	2	Metabolism
Q9CZ13	Cytochrome b-c1 complex subunit 1,mitochondrial	2	Metabolism
P08113	Endoplasmin	2	Biosynthesis
Q99LC5	Electron transfer flavoprotein subunit alpha, mitochondria	2	Metabolism
Q64467	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	2	Metabolism
P17156	Heat shock 70 kDa protein 2	2	Signal transduction
Q61847	Meprin A β-subunit	2	Inflammation
Q01853	Transitional endoplasmic reticulum ATPase	2	Protein degradation

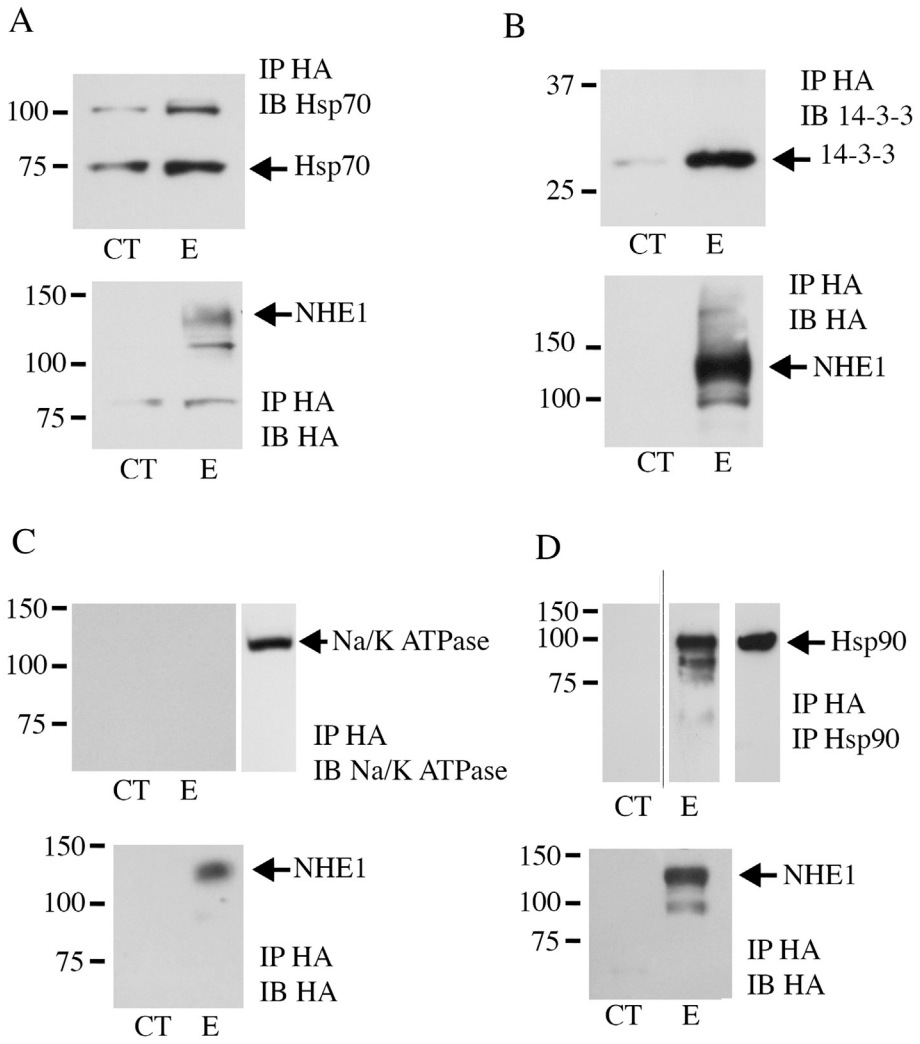

Fig. 1. Co-immunoprecipitation of HA-NHE1 and associated proteins. NHE1 was immunoprecipitated from MDCK cells with transfected with HA-tagged NHE1 protein (experimental, E) or from MDCK cells without the HA-NHE1 protein (control, CT). After immunoprecipitation with rabbit polyclonal antibody, samples of the control and experimentals were immunoblotted with antibodies against Hsp70, 14-3-3, Na^+/K^+-ATPase and Hsp90 (Fig. A–D respectively). Upper panels, immunoblots using antibody against putative co-immunoprecipitating proteins indicated. Lower panel, immunoblot against HA tag using monoclonal anti-HA antibody to confirm the immunoprecipitation of the HA-NHE1 protein. Adjacent lanes at right are cell lysates used as a positive control where necessary. The co-IP complex was solubilized with SDS-PAGE sample buffer for 30 min at 37 °C. After SDS-PAGE proteins were transferred onto nitrocellulose for immunoblotting (IB) which was done with anti-HA tag antibody or with antibody indicated. Arrow denotes location of NHE1 protein or other proteins indicated. Results are typical of at least 3 experiments.

considered to be statistically significant.

3. Results

3.1. Determination of renal NHE1 interacting partners by affinity chromatography

We produced and purified the NHE1-GST C-terminal fusion protein as described earlier [21]. This was used for affinity chromatography of rat kidney extracts. Affinity chromatography with GST alone, was used as a control. When we used affinity chromatography with GST and GST-NHE1 fusion protein, we obtained a number of interacting proteins. Table 1 summarizes the interacting proteins that were unique to the NHE1-GST and not present in the proteins eluted from GST affinity chromatography. Four of these, HSP70, HSP90, 14-3-3 and Na^+/K^+-ATPase have been suggested to be in association with the NHE1 protein earlier [22,30,31]. To examine if these proteins were in association with NHE1 in live cells we used co-immunoprecipitation experiments. Fig. 1 illustrates typical results. The NHE1 protein was immunoprecipitated from MDCK cells using anti- HA tag antibody. A mock immunoprecipitation was done from MDCK cells that did not express the tagged NHE1 protein. Fig. 1A illustrates that Hsp70 immunoprecipitates with the NHE1 protein. There was evidence of Hsp70 in the control immunoprecipitate, however it was increased an average over 3-fold ($n = 5$, $P < 0.01$) in the immunoprecipitation from MDCK cells transfected with HA tagged NHE1. Immunoblotting with anti HA antibody confirmed that the immunoprecipitation was successful. Hsp70 was present at an apparent molecular weight of 70 kDa and a dimer was also present at a size of approximately 140 kDa. Fig. 1B illustrates a similar experiment with 14-3-3 protein. There was a clear association of 14-3-3 with the immunoprecipated NHE1 protein. Despite repeated attempts, under varying conditions, we were unable to co-immunoprecipitate Na^+/K^+-ATPase with NHE1 (Fig. 1C). Using Hsp90 anti- beta isoform antibody we also co-immunoprecipitated Hsp90 protein (Fig. 1D).

3.2. 14-3-3 binding to NHE1

Acidosis is associated with hypoxia [32]. We have earlier shown that sustained intracellular acidosis activates NHE1 activity in these cells through phosphorylation dependent mechanisms [18]. 14-3-3 binding to NHE1 has also been shown to affect activity of the NHE1

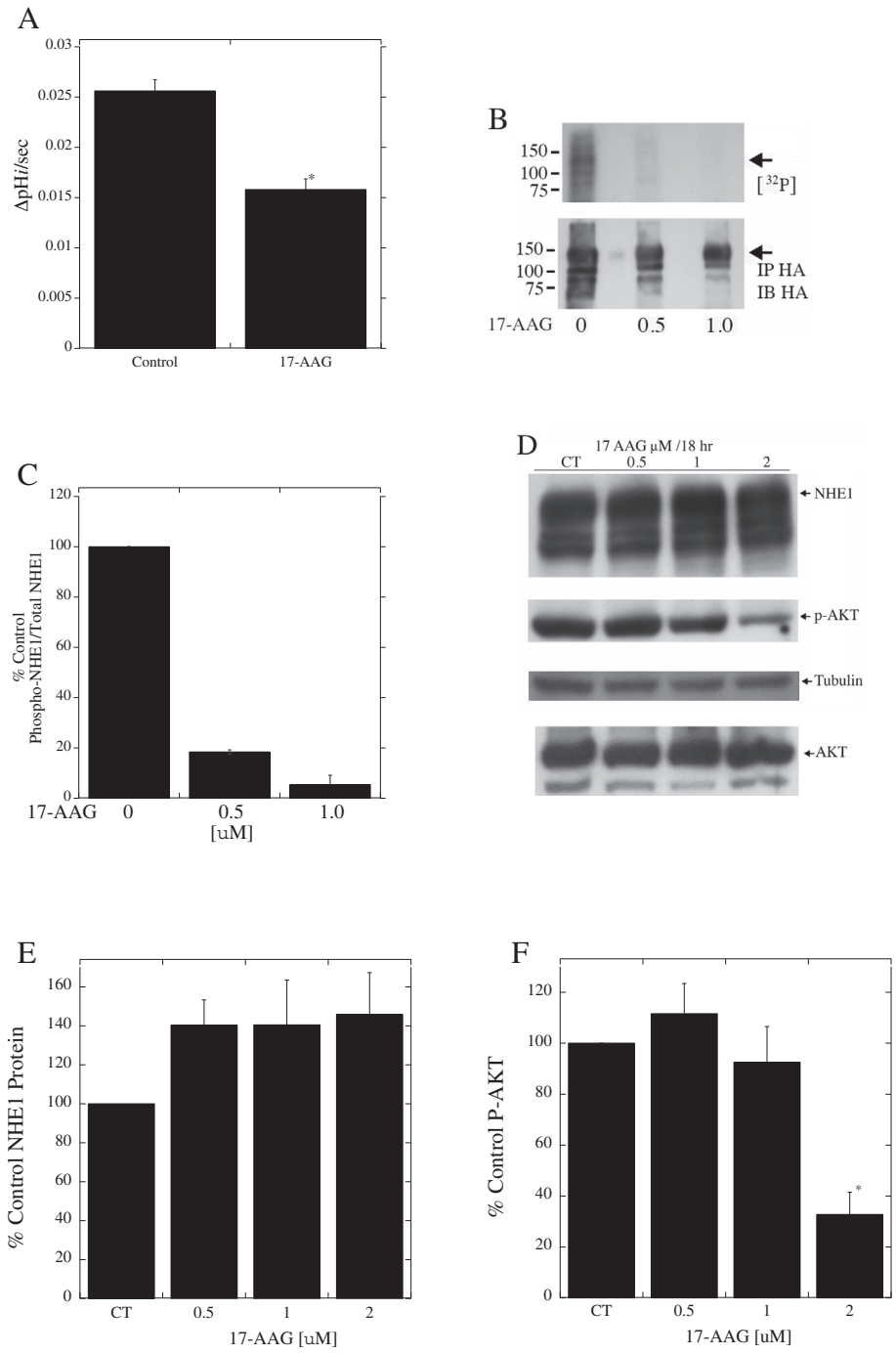

Fig. 2. Effect of 17-AAG treatment on MDCK cells. A, Effect on NHE1 activity. MDCK cells were treated with 1 μM 17-AAG for 18 h. NHE1 activity was measured as described in the "Materials and Methods". * significantly decreased from control value at $P < 0.001$, $n = 10$. B, C, Effect of 17-AAG on NHE1 phosphorylation in MDCK cells. Cells were incubated in the presence of 0, 0.5 or 1 μM 17-AAG for 18 h prior to examination of phosphorylation levels of the NHE1 protein. B, example of effect of 17-AAG on NHE1 phosphorylation, C, summary of results of C, $n = 4$. D, E effect of 17-AAG on NHE1 protein and phospho AKT levels. Cells were treated with 17 AAG for 18 h at the indicated concentrations. D, Upper panel, immunoblot for NHE1 protein using anti NHE1 antibodies. Second panel, immunoblot against phospho-AKT protein. Third panel, immunoblot against tubulin, used as a loading control. Lower panel, immunoblot against Akt protein. E, Summary of NHE1 protein levels with 17-AAG treatment. F, Summary of the effect of 17-AAG on phospho AKT levels with treatment for 18 h. $N = 4$, * significantly different at $P < 0.05$.

protein [30]. To examine if 14-3-3 binding to the NHE1 protein varied with sustained intracellular acidosis we treated cells and examined binding to NHE1. The results, (not shown) showed that there was no change in the level of 14-3-3 binding with sustained intracellular acidosis.

3.3. Heat shock protein regulation of NHE1

To examine the role of heat shock proteins in NHE1 activity we used the compound 17-AAG which is an inhibitor of Hsp90. Fig. 2A shows the effect of 17-AAG on NHE1 activity in MDCK cells. Treatment of cells with 17-AAG resulted in approximately a 40% decrease in NHE1 activity.

To determine the mechanism by which 17-AAG decreased NHE1

activity we examined the level of phosphorylation of the NHE1 protein in MDCK cells. Cells were incubated with [^{32}P] containing medium. MDCK cells had the NHE1 protein with a HA tag and this was used to immunoprecipitate out NHE1 and examine the level of phosphate incorporation into the protein. The results (Fig. 2B, C) showed that phosphorylation levels of NHE1 decreased dramatically with 17-AAG treatment. The effect was dramatic, with only low levels of NHE1 phosphorylation evident with treatment with 0.5 μM 17-AAG. We confirmed (Fig. 2B) that the NHE1 protein was immunoprecipitated from treated cells in comparable amounts to untreated cells. NHE1 was present as a larger molecular weight band over 100 kDa which likely represents a fully glycosylated HA-tagged protein, and as smaller molecular weight forms which may be unglycosylated protein as reported earlier [33], or possibly as degradation products. To ensure that 17-AAG was not affecting NHE1 protein expression we examined the level of the NHE1 protein using western blotting against the protein (Fig. 2D,E). The results confirmed that 17-AAG did not decrease the level of NHE1 protein.

3.4. AKT regulation of NHE1

Hsp90 has been shown to complex with the kinase AKT and modulate its phosphorylation [34,35]. We therefore reasoned that treatment with 17-AAG might have an effect on activation of AKT which is mediated by its phosphorylation. Fig. 2D, F, show an example and a summary of the effects of 17-AAG on phospho-AKT levels. Treatment with 17-AAG resulted in a decrease in the phospho-AKT levels at higher concentrations. There was no effect on the levels of AKT protein itself (lower panel).

To examine the role of AKT more directly, we used the highly selective AKT inhibitor MK2206 [36]. Initially we characterized the effect of MK2206 on NHE1 expression and on phospho-AKT levels in MDCK cells. The results (Fig. 3) showed that there was no significant effect on levels of the NHE1 protein (Fig. 3A,B). β-tubulin served as a control for protein loading. When phospho-AKT levels were examined we found that they were greatly reduced by this treatment (Fig. 3A,C). There was no effect on the level on the Akt protein (lower panel). There was a significant decrease in the activity of the NHE1 protein with treatment with MK-2206 (Fig. 3D). To examine if there was an additive effect of the addition of 17AAG and MK2206 we treated cells simultaneously with the two compounds and measured NHE1 activity. The results showed that there was no additional inhibition by addition of the two compounds in comparison with addition of either compound individually (Fig. 3E). When we examined the effect of these compounds on resting pHi we found that either compound individually, caused slight but not significant decreases in resting pHi. When both were applied simultaneously, there was a slight, significant decrease in resting intracellular pH (Fig. 3F). Finally, we determined if MK-2206 could interfere with the interaction between NHE1 and Hsp90. Cells were treated with MK2206 and HA-tagged NHE1 protein was immunoprecipitated. The levels of co-immunoprecpitating Hsp90 protein were quantified with correction for the amount of NHE1 protein precipitated. The results showed that treatment with MK-2206 results in a significant decrease in the amount of Hsp90 in association with the NHE1 protein.

4. Discussion

NHE1 is a critical pH regulatory protein in many tissues. In the kidney, it has been shown to be upregulated in response to acidosis and is thought to be important in pH regulation in these cells [37,38]. We recently demonstrated that in renal cells, a cluster of phosphorylatable amino acids in the regulatory tail are important in mediating NHE1 activation through Erk-dependent pathways [39]. However, many regulatory proteins of NHE1 have been shown to bind to the NHE1 C-terminus [7] and activation of the protein is thought to be mediated through a combination of both phosphorylation and protein mediated interactions on the regulatory C-terminus. We therefore examined proteins interacting with NHE1 in the mammalian kidney. Our initial screen used the cytoplasmic regulatory domain of NHE1 to identify NHE1 binding proteins from kidney extracts. We identified a number of proteins from kidney extracts (Table 1) that bound to the cytosolic domain. Among them, some with higher scores were heat shock proteins, 14-3-3 proteins, and Na^+/K^+ ATPase.

4.1. NHE1 interactions with the cytosolic domain, Na^+/K^+ ATPase

We chose several of these to investigate further, based on their higher score and on previous publications. It has earlier [31] been suggested that NHE1 and Na^+/K^+ ATPase interact in a complex. For this reason we attempted to confirm the interaction demonstrated in our screen of proteins bound to the NHE1 tail. Nevertheless, despite repeated attempts including cross linking of proteins, we were unable to immunoprecipitate Na^+/K^+ ATPase with NHE1. The reason for this is not yet apparent but could be due to a transient interaction of the two proteins or perhaps an interaction that is regulated, and was not apparent under the conditions of the assay we used. Nevertheless, our demonstration of an association using the cytosolic domain of NHE1, when taken together with the previous published report [31] suggests that there is an interaction, perhaps transient in nature.

4.2. NHE1 interactions with the cytosolic domain, 14-3-3 proteins

The 14-3-3 proteins have been demonstrated to interact with the NHE1 tail. They regulate phosphorylation of serine 703 of NHE1 in the myocardium [30,40]. However, while 14-3-3 regulation of NHE1 has been demonstrated in the myocardium there has been little study of it in other tissues. We found that 14-3-3 both immunoprecipitated with NHE1 from renal cells, and also bound to the NHE1 C-terminal protein *in vitro*. We had previously demonstrated that sustained intracellular acidosis stimulated NHE1 activity [18]. We therefore examined if 14-3-3 might be involved in this phenomenon. However, there was no change in the level of 14-3-3 binding with sustained intracellular acidosis. 14-3-3 binds to and regulates phosphorylation of serine 703. These results are therefore in agreement with our previous results [41] which suggested that sustained acidosis activates NHE1 independent of serine 703. The role of 14-3-3 in regulation of NHE1 in the kidney is unresolved at this time. Experiments with treatment of MDCK cells with aldosterone, failed to show any change in the level of 14-3-3 association with NHE1 (not shown). Future experiments may address this phenomenon.

4.3. NHE1 interactions with the cytosolic domain, heat shock proteins and AKT

The heat shock proteins Hsp70 and Hsp90 of kidney cell extracts were found to associate with NHE1 *in vitro* (Table 1). Initial attempts at immunoprecipitation of these two proteins with NHE1 were successful for Hsp70 and for Hsp90. There have been earlier reports of an association of heat shock proteins with NHE1. An association was demonstrated earlier [22] by both affinity chromatography and by immunoprecipitation from PS127A cells. More recently [42] Hsp70 was shown to interact with NHE1 dynamically in macrophages. Our results here confirm that these two heart shock proteins interact with NHE1 in kidney cell types.

Immunoprecipitation experiments showed that Hsp90 interacted with NHE1. We therefore investigated the physiological role of this interaction. To examine if there was a potential role for Hsp90 in regulation of NHE1 activity, we treated cells with 17-AAG which is a Hsp90 inhibitor [43]. Treatment with 17-AAG resulted in a decrease in NHE1 activity and there was also a large decrease in the level of NHE1 phosphorylation (Fig. 2). NHE1 contains phosphorylation sites for a

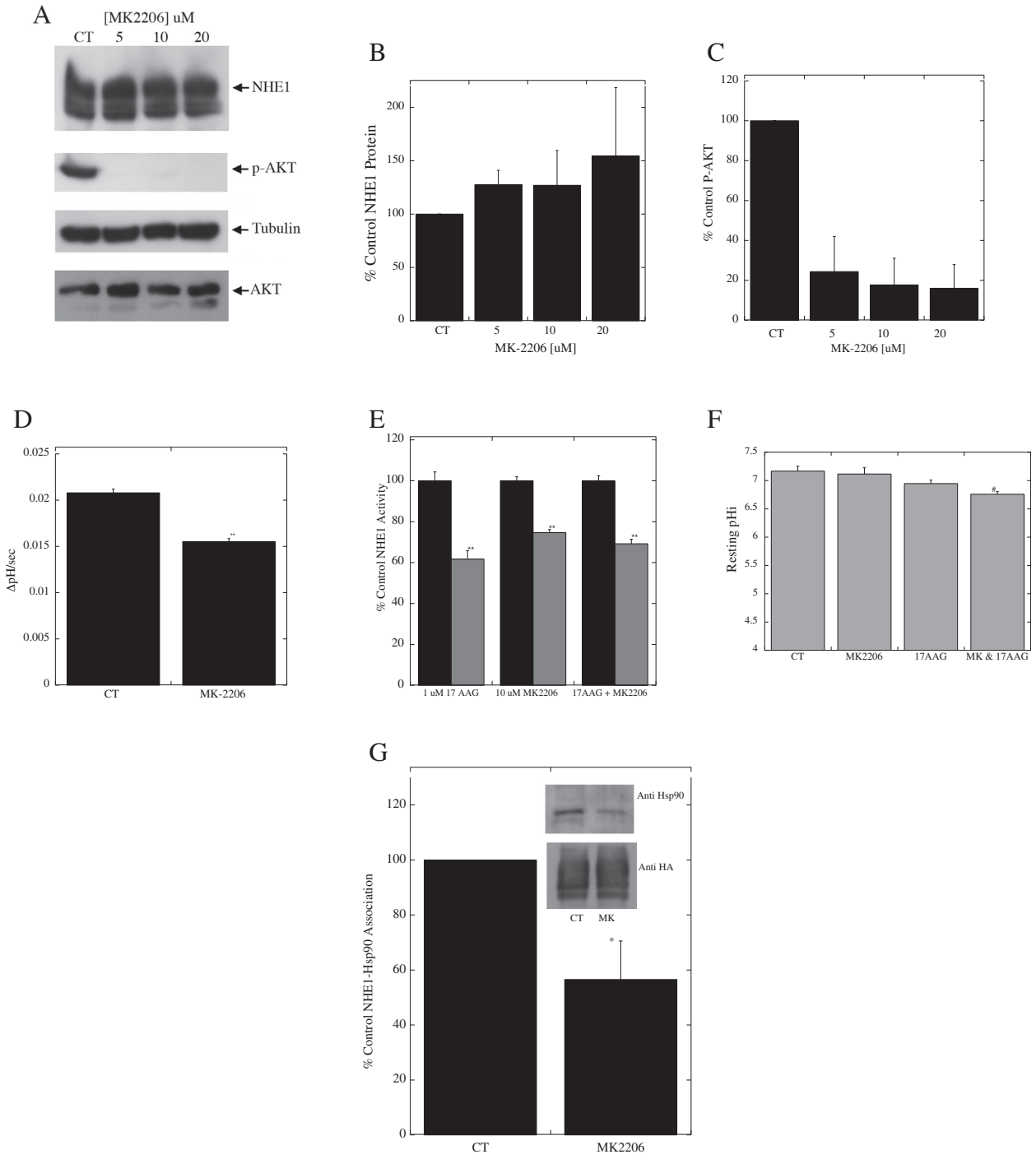

Fig. 3. Effect of MK-2206 treatment on MDCK cells. A, Cells were treated with MK-2206 for 18 h at the indicated concentrations. Upper panel, immunoblot for NHE1 protein using anti-NHE1 antibodies. Second panel, immunoblot against phospho-AKT protein. Third panel, immunoblot against tubulin, used as a loading control. Lower panel, immunoblot against Akt protein. B, Summary of effect of MK-2206 on NHE1 protein levels. MK-2206 was used at the indicated concentrations for 18 h. C, Summary of effect of MK-2206 on phospho-AKT levels. D, Effect of MK-2206 treatment (10 μM × 18 h) on NHE1 activity of MDCK cells. $N = 10$, ** significantly different at $P < 0.001$. E, effect of combinatorial treatment of 17AAG and MK-2206 on NHE1 activity, in comparison with the effect of individual treatments. NHE1 activity was measured as described above. MK2206 and 17-AAG were applied as above. $N = 10$. F, effect of individual or combinatorial treatment of 17AAG and MK-2206 on resting pHi of MDCK cells. Resting pHi was measured as described above. MK2206 (10 μM × 18 h) and 17-AAG (1 μM × 18 h) were applied as above. $N = 6$–9. # significantly different at $P < 0.01$. G, Effect of MK2206 treatment (10 μM × 18 h) on co-immunoprecipitation of Hsp90 with NHE1. NHE1 was immunoprecipitated from MDCK cells as described in Fig. 1 using anti-HA antibodies. Co-immunoprecipited Hsp90 was quantified by western blot analysis with controls compared to MK2206 treated cells. Results were corrected for the amount of NHE1 in immunoprecipitate. $N = 6$, * significantly different at $P < 0.05$. Inset, upper panel, example of western blot of co-immunoprecipitating Hsp90 from control (CT) and MK2206 (MK) treated cells. Lower panel, immunoblotting for NHE1 with anti-tag (HA) antibody, to measure levels of NHE1 in immunoprecipitate.

number of protein kinases [7,28]. So the almost total disappearance of phosphorylation of NHE1 by treatment with 17-AAG was somewhat surprising, and could suggest that the activity of several different protein kinases was affected. Alternatively, AKT phosphorylation may represent much of the phosphorylation present in MCT cells. In addition to 17-AAG, the selective AKT inhibitor MK-2206 decreased NHE1 activity and Phospho-AKT levels, supporting a role for AKT regulation mediated through Hsp90. It was noteworthy that addition of 17-AAG

and MK-2206 together, did not result in an additive inhibitory effect on NHE1 activity, suggesting they act through the same mechanism. When both compounds were applied together, there was a slight, significant decrease in resting pHi of MDCK cells. Resting pHi would certainly be influenced by NHE1 activity, but other pH regulatory proteins could also be exerting an influence on the resting pHi. Of note, treatment of cells with MK-2206 resulted in a decrease in the level of Hsp90 associated with NHE1. AKT forms a regulatory complex with Hsp90 [35]. A decrease in the association of this complex with NHE1, could reduce the ability of AKT to phosphorylate NHE1.

It was therefore apparent that in our system, Hsp90 can act to regulate NHE1 activity. Inhibition of Hsp90 and AKT resulted in a decrease in phosphorylation of NHE1 and decreased activity. This would suggest that AKT has a stimulatory role on NHE1 in kidney cells. In previous reports AKT phosphorylation of NHE1 has been shown to be either stimulatory or inhibitory. In cardiac tissue, the activity of the NHE1 protein was inhibited by AKT mediated phosphorylation of serine 648 [44,45]. However, another report studied fibroblasts and showed that AKT phosphorylation of NHE1 is stimulatory and is necessary for activation of NHE1 by insulin and PDGF [46]. It has been suggested that the exact role of AKT mediated phosphorylation of NHE1 may depend on cell type [7]. AKT phosphorylation of NHE1 occurs at serine 648 which overlaps with the first calmodulin binding site of the autoinhibitory domain. Snabaitis et al. [44] demonstrated that phosphorylation of serine 648 inhibits calmodulin binding to this domain. We have demonstrated that calmodulin interaction with NHE1 is dynamic *in vivo* in fibroblasts, increasing in response to calcium and hormonal stimulation [47]. In the myocardium, calcium levels are key to contractility and rise to relatively high levels with contraction. It may be that in myocardial cells, the elevated level of calcium interacts with calmodulin and that this, leads to a more chronic activation. AKT-mediated phosphorylation in this circumstance may be inhibitory. In other tissues, with lower calcium levels, the role of calmodulin association at the site may be reduced. Phosphorylation by AKT could be stimulatory by mediating reduced inhibition of the auto-inhibitory domain in the absence of calcium/calmodulin complex. Further studies are necessary to examine this phenomenon.

5. Conclusion

We recently examined the NHE1 interactome in triple negative breast cancer cells [48]. There were striking differences and similarities compared with what we found in the present study. Similar to the present report, heat shock proteins and 14-3-3 were found. In that study we also found associated ERM proteins and chaperones such as protein disulfide isomerase, calnexin and calreticulin. Na^+/K^+ ATPase was not found associated though several other integral channel proteins were found associated including a chloride channel and anion channel. The significance of these putative associations and their tissue specificity needs to be verified and investigated.

Our study is the first study beginning to define the NHE1 interactome in the kidney. We demonstrate that several proteins interact with NHE1 in the kidney. We show the interaction of Hsp90 with NHE1 in renal cells and that Hsp90 inhibition decreases NHE1 activity. As Hsp90 is known to regulate the activity of the kinase AKT [34,35] we examined the effect of AKT inhibition and demonstrated a role for AKT in modulating NHE1 activity which could be mediated through Hsp90-NHE1 association. Based on our results so far, we propose that AKT plays a stimulatory role in NHE1 activity in renal cells, which may be through an association of Hsp90 with NHE1.

Disclosures

There were no conflicts of interest financial or otherwise.

Author contributions

AO carried out the experiments in the laboratory and carried out data analysis. LF conceived and supervised the project and the manuscript preparation.

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