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Structural determinants for the ouabain-stimulated increase in Na–K ATPase activity

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ABSTRACT

Recent studies suggest that at low concentrations, ouabain increases Na–K ATPase and NHE1 activity and activates the Src signaling cascade in proximal tubule cells. Our laboratory demonstrated that low concentrations of ouabain increase blood pressure in rats. We hypothesize that ouabain-induced increase in blood pressure and Na–K ATPase activity requires NHE1 activity and association. To test this hypothesis we treated rats with ouabain (1 μg kg body wt^{-1} day^{-1}) for 9 days in the presence or absence of the NHE1 inhibitor, zoniporide. Ouabain stimulated a significant increase in blood pressure which was prevented by zoniporide. Using NHE1-expressing Human Kidney cells 2 (HK2), 8 (HK8) and 11 (HK11) and Mouse Kidney cells from Wild type (WT) and NHE1 knock-out mice (SWE) cell lines, we show that ouabain stimulated Na–K ATPase activity and surface expression in a Src-dependent manner in NHE1-expressing cells but not in NHE1-deplete cells. Zoniporide prevented ouabain-induced stimulation of ^{86}Rb uptake in the NHE1-expressing cells. FRET and TIRF microscopy showed that ouabain increased association between GFP-NHE1 and mCherry-Na–K ATPase transfected into NHE1-deficient SWE cells. Mutational analysis demonstrated that the caveolin binding motif (CBM) of Na–K ATPase α1 is required for translocation of both Na–K ATPase α1 and NHE1 to the basolateral membrane. Mutations in activity or scaffold domains of NHE1 resulted in loss of ouabain-mediated regulation of Na–K ATPase. These results support that NHE1 is required for the ouabain-induced increase in blood pressure, and that the caveolin binding motif of Na–K ATPase α1 as well as the activity and scaffolding domains of NHE1 are required for their functional association.

1. Introduction

Na–K ATPase plays two separate roles in cell metabolism. Its best known function is as the mediator of sodium–potassium exchange, regulating intracellular ion content and transepithelial ion movement [1,2]. This process is highly regulated and specifically inhibited by cardiac glycosides, which are used pharmacologically in the treatment of heart failure and cardiac arrhythmias [3,4]. Within the past decade, a second function for Na–K ATPase has been described, as it being a receptor for cardiac glycoside-stimulated signal transduction through an EGFR/Src/ERK-dependent pathway [4–7]. Whether cardiac glycoside stimulation results in Na–K exchange or activation of ERK appears to be a function of the concentration of the cardiac glycoside, in that the pharmacologic doses used for treatment of heart failure inhibit ion exchange, whereas much lower physiologic concentrations actually stimulate ion exchange and the EGFR/Src/ERK signaling pathway [8,9]. The physiologic role for cardiac glycoside-stimulated sodium pump activity remains unclear. Furthermore, this function may also be specific to some but not all Na–K isoforms.

We have recently demonstrated that low concentrations of ouabain, a well-studied cardiac glycoside, stimulate Na–K ATPase-mediated ATPase activity, ^{86}Rb uptake, and Na–K ATPase α1 subunit phosphorylation and trafficking to the basolateral membrane of renal proximal tubule cells [10]. Low-dose ouabain also activates a signaling pathway involving Src, ERK, and Akt that promotes cell proliferation [11]. Interestingly, these effects were seen in renal proximal tubule cell culture models that expressed NHE1 (HKC-5, HK11, and OK cells), but not in HK2 cells, a cell culture model of proximal tubule with a very low expression of NHE1 [10]. Similarly, inhibition of NHE1 activity with ethyl-isopropyl amiloride (EIPA) blunted the ouabain-stimulated

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increase in Na–K ATPase activity. We further demonstrated that treatment with low-dose ouabain increased basolateral membrane expression and stimulated increased association of NHE1 with the Na–K ATPase α1 subunit [10].

NHE1 is a ubiquitously expressed member of the NHE family implicated in the regulation of multiple processes including intracellular pH [12,13], cell size [14,15], and cell proliferation [16,17]. In the renal proximal tubule, NHE1 expression is confined to the basolateral membrane [12], the same distribution as Na–K ATPase. NHE1 contains two well characterized domains, an N terminal activity domain responsible for sodium–hydrogen exchange and a C terminal scaffolding domain for protein interactions [18]. Our previous studies suggested that at least the activity domain of NHE1 is required for ouabain-stimulated Na–K ATPase activity and signal transduction [10]. The demonstration that low-dose ouabain stimulates increased association of NHE1 with Na–K ATPase α1 subunit suggests the possibility that the scaffolding domain is also required.

Based on these findings, we hypothesized that ouabain-stimulated NHE1–Na–K ATPase α1 subunit association is dependent upon both the activity and scaffolding domains of NHE1. The purpose of this study was to determine the sites on both proteins required for their interaction and function. Our data show that both the activity and scaffolding domains of NHE1 are required for cardiac glycoside stimulation of Na–K ATPase activity, and indicate that the caveolin binding motif of the Na–K ATPase α1 subunit is critical for basolateral membrane localization of both Na–K ATPase and NHE1. We further demonstrate that the effects of low-dose ouabain are in fact dependent on the activation of the Src signaling pathway.

2. Experimental procedures

2.1. Materials

Ouabain and zoniporide were purchased from Tocris (St. Louis, MO). Digibind was purchased from GlaxoSmithKline (Parma, Italy). Antibodies against caveolin-1 were purchased from Novus Biological (Littleton, CO). The monoclonal antibody against Na–K ATPase α1 (α6F), developed by Dr. D.M. Fambrough, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of NIHCD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Monoclonal antibodies against NHE1 were purchased from BD Biosciences (San Jose, CA). HRP-linked secondary antibodies were purchased from Vector laboratories. Streptavidin agarose resin was purchased from Pierce Biotechnology (Rockford, IL). Phosphatase inhibitor cocktail-1 and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). All other chemicals were purchased from Sigma, unless otherwise specified.

2.2. Animal model

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Louisville.

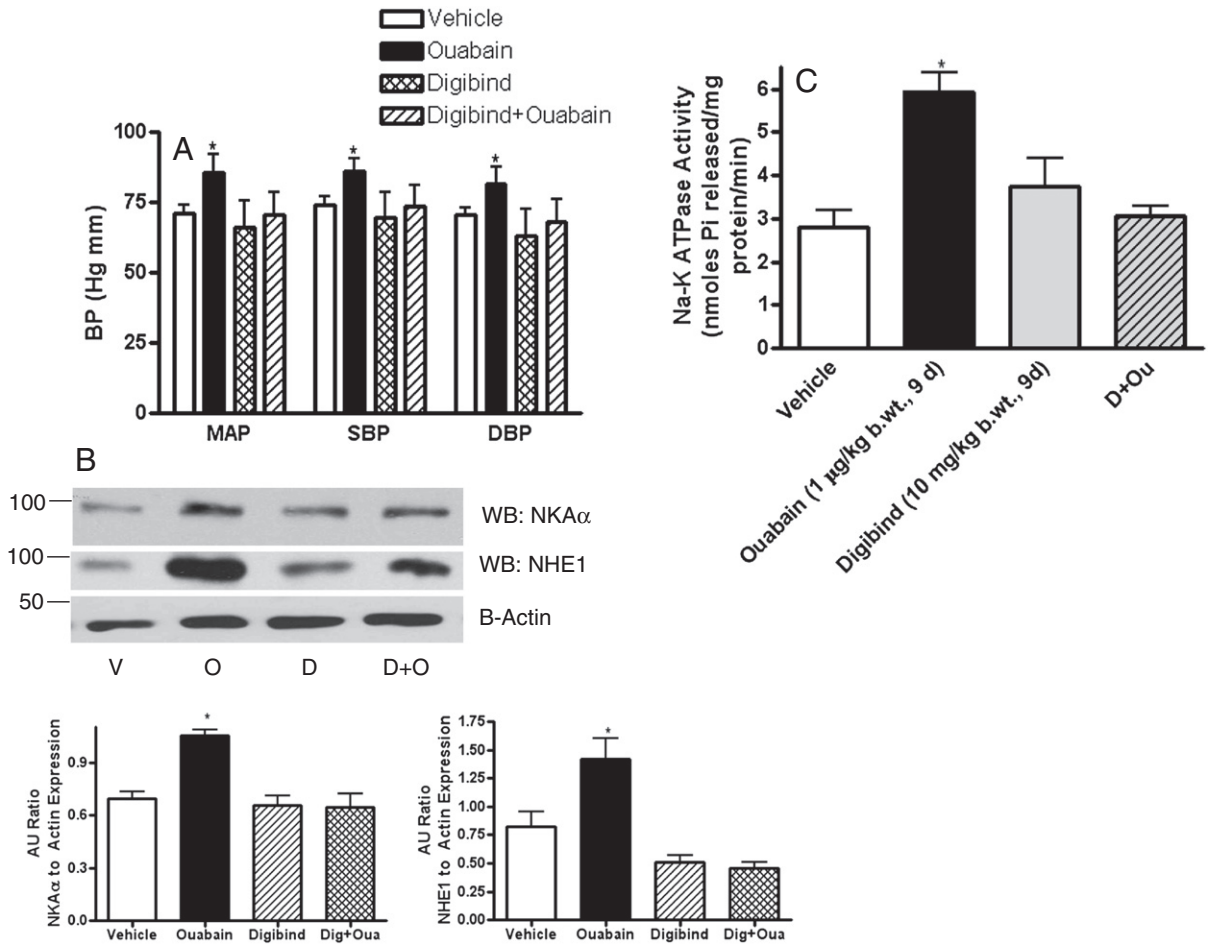


Fig. 1. Effect of digibind on ouabain-induced blood pressure. A, Each bar represents blood pressure as mean ± se (n = 6 in each group) in male Sprague Dawley rats treated with ouabain (1 μg kg body wt.$^{-1}$ day^{-1}) for 9 days in the presence or absence of digibind (10 mg kg body wt.$^{-1}$ day^{-1}). B–D, Expression of Na–K ATPase α1 and NHE1 (B), or Na–K ATPase activity (C) in kidney BLM preparations from the above rat groups. Data are expressed as mean ± se from six individual animals (n = 6) in each group. *Indicates $P < 0.05$ from vehicle-treated animal groups as calculated by one-way ANOVA followed by Bonferroni's analysis. Right kidneys were fixed in 3.7% paraformaldehyde and embedded in paraffin. Representative IHC for Na–K ATPase α1 (D) and NHE1 (E) is shown (n = 6). All pictures were taken using a 40× objective.

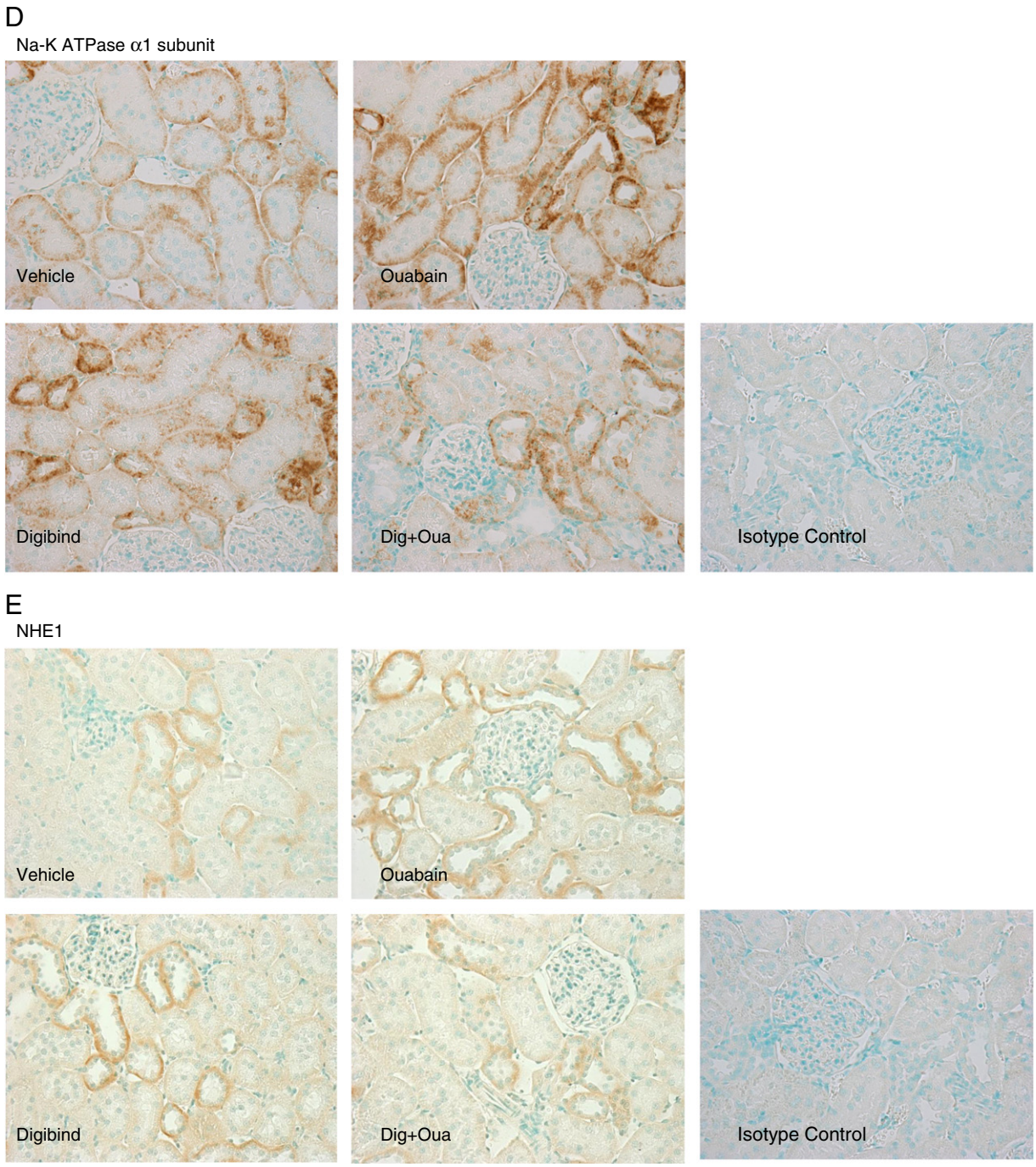


Fig. 1 (*continued*).

Male Sprague Dawley rats weighing 200–250 g were stabilized on the standard rat chow and water ad libitum for a week prior to experiments. Animals were divided into 2 sets of 4 groups (6 animals in each group). In each set group A received PBS intraperitoneally (control), group B received ouabain (1 μg kg body wt^{-1} day^{-1}), group C received digibind (10 mg kg body $wt.^{-1}$ day^{-1}) or zoniporide (1 mg kg body $wt.^{-1}$ day^{-1}), and group D received both digibind and ouabain or both zoniporide and ouabain for 9 days. Treatment with digibind or zoniporide was started on the day of ouabain treatment. Blood pressure was measured in ketamine-anesthetized rats after 9 days of treatment by placing a catheter in the right carotid artery and data were analyzed by using customized Micro-Med software as described previously [10]. The animals were sacrificed and kidneys were removed, decapsulated, and collected in ice-cold PBS (left kidney) for basolateral membrane (BLM) preparation or 3.7% paraformaldehyde (right kidney) for immunohistochemistry.

2.3. Basolateral membrane isolation

Kidney cortical BLMs were prepared from the above group of rats by the method of Sacktor et al. [19] as described previously [10].

2.4. ATP hydrolysis assay

BLM vesicles were quickly frozen in liquid nitrogen and slowly thawed on ice to make them permeable to ATP prior to measurement

of Na–K ATPase activity. Na–K ATPase activity in basolateral membranes was assayed as ouabain (4 mM)-sensitive ATP hydrolysis as previously described [20]. The inorganic phosphate released was measured as described previously [20].

2.5. Immunohistochemistry

Immunohistochemistry of kidney slices (3 μm) from the rats described above was performed as described previously using polyclonal antibodies against rat Na–K ATPase α1 subunit, NHE1, or appropriate isotype control IgG (negative control) [10].

2.6. Cell culture

Human kidney proximal tubule cells HK2 (ATCC), HK8, and HK11 (a gift from Dr. Lorraine Racusen, Johns Hopkins University Baltimore, MD), or mouse renal proximal tubule cells from wild-type (WT) and NHE1 knock-out (SWE) animals (a gift from Dr. Jeffery Schelling, Case Western University, Cleveland, OH) were cultured as previously described [9]. Cells were maintained in DMEM-F12 (1:1) supplemented with 10% FBS and 1% penicillin/streptomycin, and cultured to 90–95% confluence. Cells were washed with serum-free medium 24 h before use. Unless otherwise stated, human kidney cells were treated for 15 min with 10 pM ouabain and mouse kidney cells were treated for 15 min with 100 nM ouabain.

2.7. Ouabain-sensitive ^{86}Rb uptake

Ouabain-sensitive ^{86}Rb uptake in cultured cells was measured at 37 °C exactly as described previously [9,11].

2.8. Western blot analysis

Western blot was performed exactly as described previously [9,11].

2.9. Biotinylation

Surface biotinylation was performed as described previously [21]. Briefly, cells were treated with ouabain for 15 min, washed with cold PBS, and incubated with N-hydroxysulfosuccinimidobiotin (10 μl/ml) in borate buffer, pH 9.0 (20 mM Tris, 150 mM NaCl, 10 mM boric acid, 7.2 mM KCl, 1.8 mM $CaCl_2$) for 2 h at 4 °C. Cells were then washed 3 times with cold PBS, quenched with 100 mM glycine in PBS for 15 min at 4 °C, and then washed 3 times more. Next, crude membranes were isolated and resuspended in buffer containing 300 mM Mannitol, 5 mM Tris–HCl pH 7.4, 0.1% Triton X-100, and 1% (vol/vol) protease and phosphatase inhibitor cocktails. Crude membranes were incubated with streptavidin agarose resins overnight on a rotator at 4 °C. Proteins bound to the resins were eluted in 1× Laemmli sample buffer and then resolved on 10% SDS-PAGE followed by immunoblotting using monoclonal antibodies against Na–K ATPase α1 subunit (α6F) or NHE1.

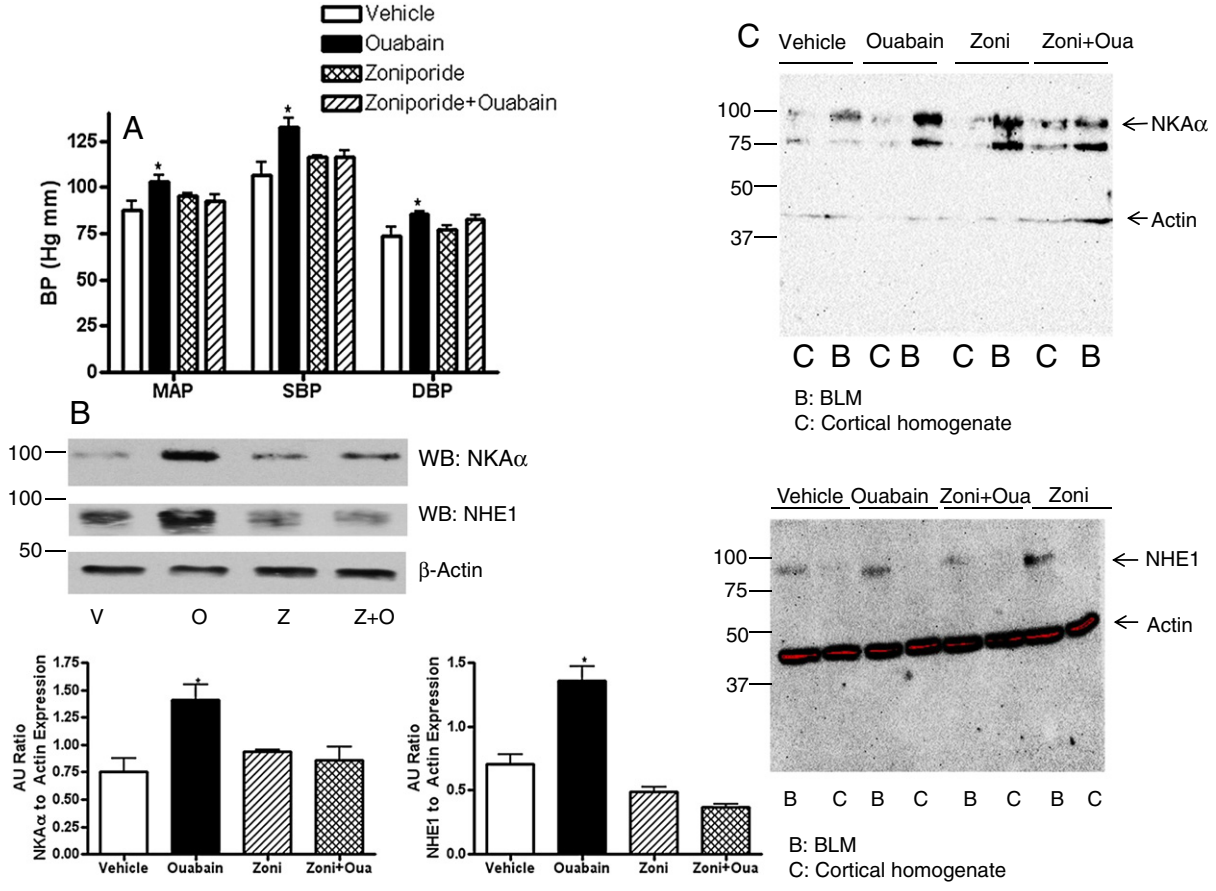


Fig. 2. Effect of zoniporide on ouabain-induced blood pressure. A, Each bar represents blood pressure as mean ± se (n = 6 in each group) in male Sprague Dawley rats treated with ouabain (1 μg kg body wt.$^{-1}$ day^{-1}) for 9 days in the presence or absence of zoniporide (1 mg kg body wt.$^{-1}$ day^{-1}). B–D, Expression of Na–K ATPase α1 and NHE1 (B and C), and Na–K ATPase activity (D) in kidney BLM preparation from the above rat groups. (E) Representative western blots for NHE1 or Na–K ATPase α1 subunit in kidney BLM preparations from the above animal groups immunoprecipitated using anti-Na–K ATPase α1 or anti-NHE1, respectively. Data are expressed as mean ± se from six individual animals (n = 6) in each group. *Indicates $P < 0.05$ from vehicle-treated animal groups as calculated by one-way ANOVA followed by Bonferroni analysis. Right kidneys were fixed in 3.7% paraformaldehyde and embedded in paraffin. Representative IHC for Na–K ATPase α1 (F) and NHE1 (G) is shown (n = 6). All pictures were taken using a 40× objective.

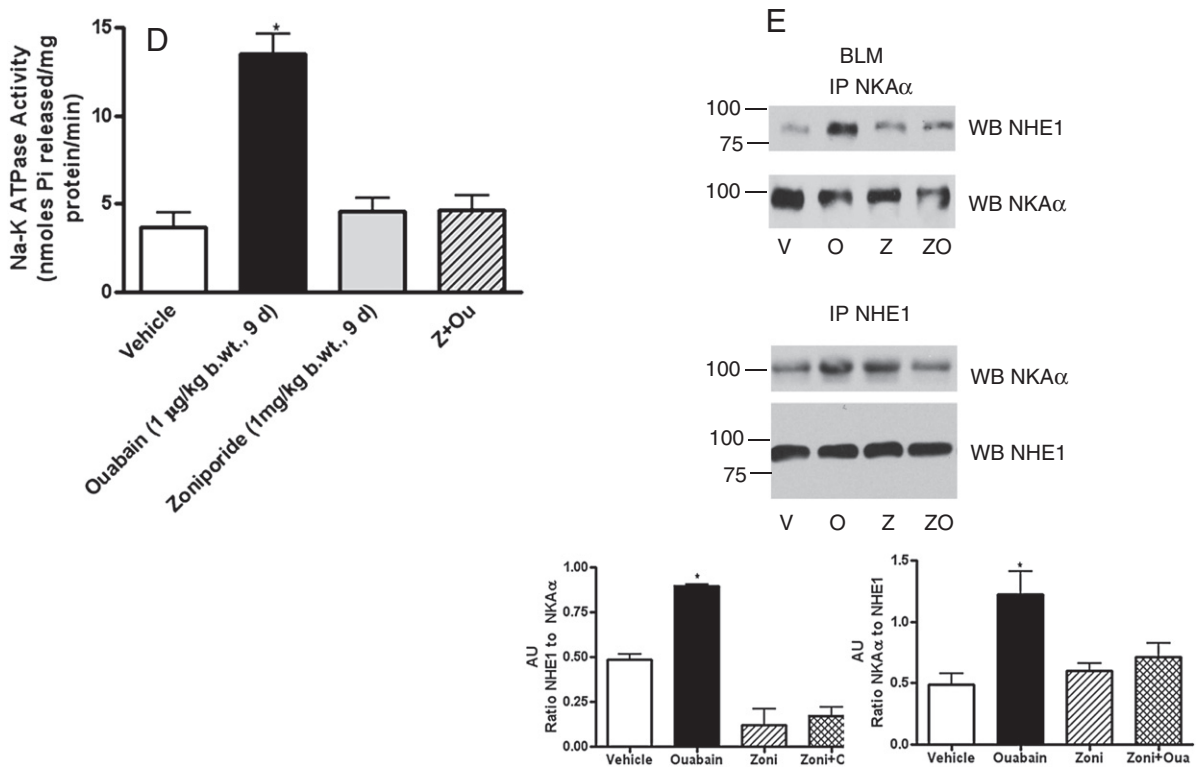


Fig. 2 (*continued*).

2.9.1. Plasmid constructs

GFP- or RFP-tagged NHE1 was a kind gift from Dr. Yung-Hsiang Yi (National Yang-Ming University, Taipei, Taiwan) [22]. mCherry-tagged rat Na–K ATPase α1 was provided by Dr. Thomas A. Pressley and YFP-tagged caveolin binding motif (CBM)-mutated (Phe^{92}-XXXX-Phe^{97}-XX-Phe^{100} to Phe^{92}-XXXX-Ala^{97}-XX-Ala^{100}, F97A/F100A) Na–K ATPase α1 was provided by Dr. Zijian Xie [23]. HA-tagged NHE1 full-length, activity-deficient mutant (E266I), and scaffold-deficient mutant (KR/A) [18] were kindly provided by Dr. Dianne Barber, UCSF.

2.9.2. Transfection

SWE cells were transfected with the indicated plasmids using Lipofectamine-2000 (Invitrogen) in OPTI-MEM as described previously [11].

2.9.3. Sensitized FRET

Försters' Resonance Energy Transfer (FRET) imaging experiments were performed in living cells. SWE cells were transfected with GFP-NHE1 (donor) and mCherry-Na–K ATPase α1 (acceptor) for 24 h and then viewed and analyzed with an Olympus FRET/TIRF microscope (Center Valley, PA). FRET image acquisition and analysis were performed using SlideBook software version 4.2 as described previously [24]. The software is based on the three-filter "micro-FRET" image subtraction method described by Jiang and Sorkin [25]. Briefly, three images (100-ms or 250-ms exposure sets, 2 × 2 binning) were obtained: an mCherry excitation/mCherry emission image; a GFP excitation/GFP emission image; and a GFP excitation/mCherry emission image (raw, uncorrected FRET). After imaging, background images were taken. Background-subtracted mCherry and GFP images were fractionally subtracted from raw FRET images based on measurements for GFP bleed-through (0.50–0.56) and mCherry cross-excitation (0.015–0.02). This fractional subtraction generates corrected FRETC images. The corrected images were represented in pseudo-color (gated to mCherry acceptor levels), showing sensitized FRET within cells. The subtraction Pearson's coefficients were rounded up from average cross-bleed values determined in cells expressing GFP- or mCherry-tagged constructs alone. Thus, these coefficients result in the underestimation of FRETC signals for true FRET partners but prevent false positive detection of FRET. Sensitized donor or acceptor normalized FRET was calculated using SlideBook4.2 software.

2.9.4. TIRF microscopy

Membrane Total Internal Reflection Fluorescence (TIRF) was performed as described by Blaine et al. [26] with slight modifications. Briefly, SWE cells were grown to 60% confluence in a dish with a collagen-coated coverslip bottom (no. 1.5, MatTek, Ashland, MA). Cells were transfected with the indicated plasmids as described in Results. Samples were observed using an Olympus TIRF microscope equipped with a 60× 1.45 numerical aperture (NA) objective under the control of SlideBook software (version 4.2, Olympus, Center Valley, PA). Laser excitation was derived from a multiline argon ion laser run at the same current setting for all experiments. The power at the sample was controlled by a neutral density filter wheel. Excitation and emission wavelengths were selected using filters set for mCherry and GFP. The laser was aligned per the manufacturer's instructions to achieve TIRF illumination. Images were taken using a Hamamatsu camera operating with 2 by 2 binning. Oxygen was provided by the ambient air, which was supplemented by 5% CO_2 and warmed to 37 °C in an environmental chamber surrounding the specimen.

2.9.5. Protein determination

Protein concentration was determined using a bicinchoninic acid protein assay kit (Sigma) using BSA standard.

2.9.6. Statistics

Data are shown as mean ± SE. The *n* values represent the number of independent experiments. Each experiment was performed in triplicate. *P* values were calculated using one-way ANOVA, followed by Bonferroni analysis using GraphPad Prism software. A *P* value <0.05 was a priori considered statistically significant.

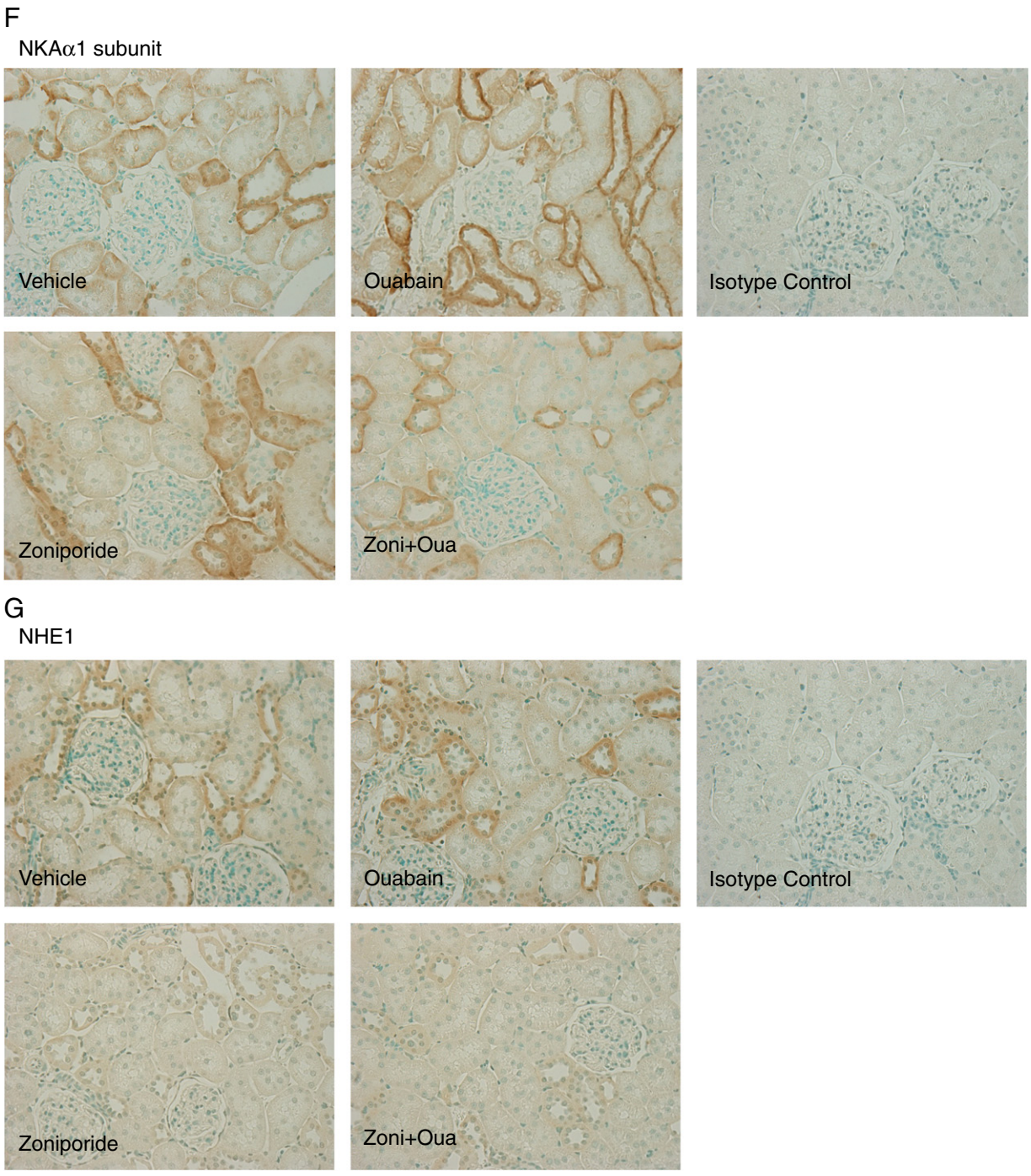


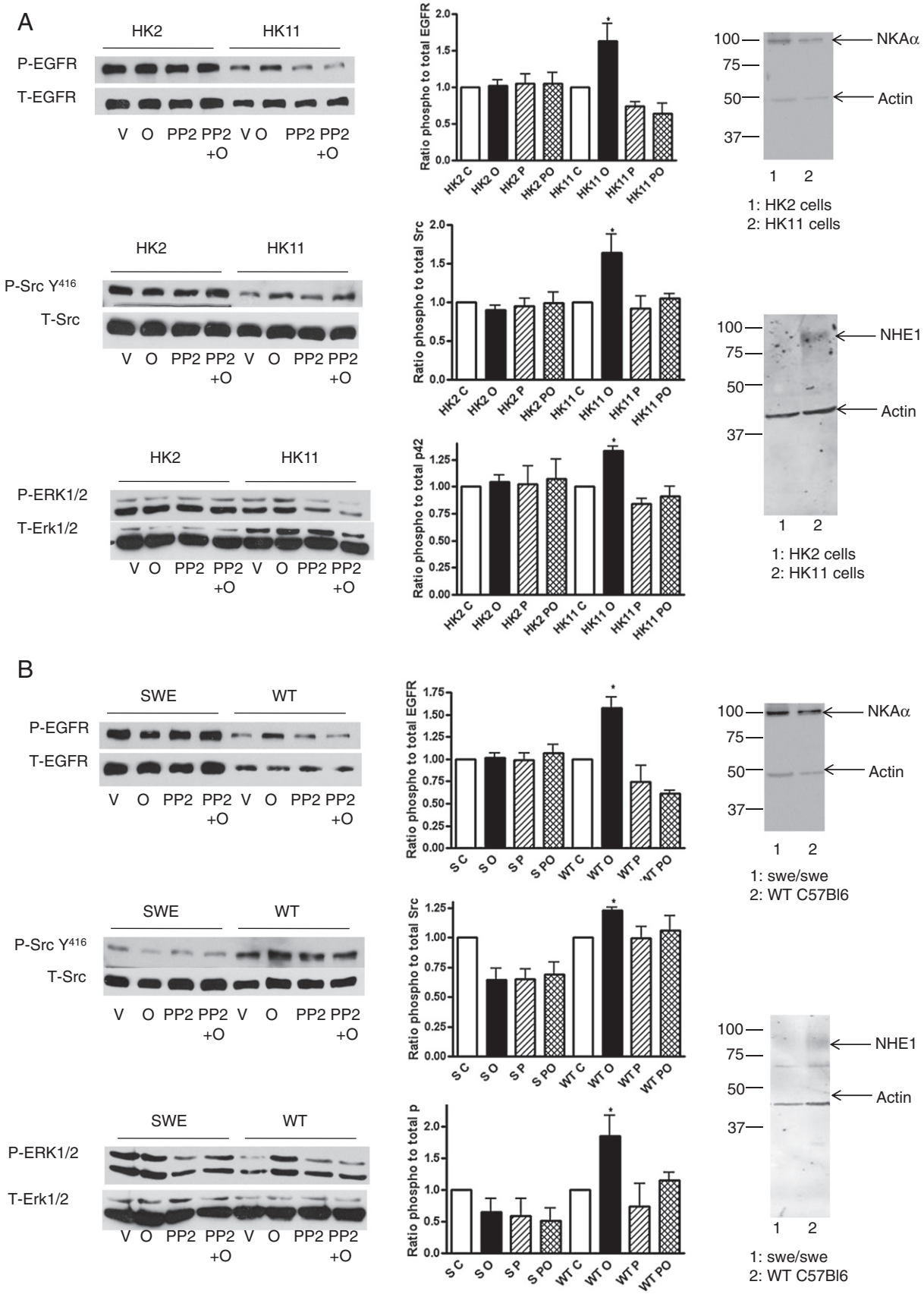
Fig. 2 (*continued*).

3. Results

3.1. Effect of ouabain on blood pressure

We have previously demonstrated that treatment of rats with low-dose ouabain increases blood pressure after 9 days [10]. To determine whether this effect is due to the cardiac glycoside properties of ouabain, we treated male Sprague–Dawley rats with ouabain in the presence or absence of digibind, an FDA-approved antibody against cardiac glycosides to treat cardiac glycoside toxicity [27]. As shown in Fig. 1A, treatment with ouabain increased blood pressure, which was prevented by treatment with digibind. Ouabain-mediated increase in Na–K ATPase α1 expression (Fig. 1B) and activity (Fig. 1C) in isolated renal tubular BLMs was also prevented by treatment

Fig. 3. Effect of NHE1 and Src kinase on ouabain-stimulated signaling in renal proximal tubule cells. Cells were treated for 15 min with 10 pM (A, HK2 and HK11 cells) or 100 nM (B, SWE and WT cells) ouabain in the presence or absence of the Src kinase inhibitor PP2 (1 μM). Cells were lysed and nuclear-free extracts were used to determine phosphorylation of EGFR (top panel), Src (middle panel), or ERK1/2 (bottom panel) by western blot using phospho-specific antibodies. The nitrocellulose membranes were stripped and reprobed using antibodies against the total protein. A representative blot is shown (n = 4). Bar diagrams show arbitrary densitometry values (mean ± se) as ratio of phospho to total expression from 4 independent experiments. The values were plotted as fold difference from the vehicle-treated group. *Indicates $P < 0.05$ as calculated by one-way ANOVA followed by Bonferroni analysis. A & B, right panels show representative immunoblots for Na–K ATPase α1 and NHE1 in crude membrane preparations from untreated HK2, HK11, WT, and SWE cells.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HK2
HK11
P-EGFR
T-EGFR
V O PP2 PP2 +O V O PP2 PP2 +O
Ratio phospho to total EGFR
HK2 C HK2 O HK2 P HK2 PO HK11 C HK11 O HK11 P HK11 PO
100 75 50 37
NKAα
Actin
1 2
1: HK2 cells
2: HK11 cells
P-Src Y416
T-Src
Ratio phospho to total Src
NHE1
P-ERK1/2
T-Erk1/2
Ratio phospho to total p42
B
SWE
WT
S C S O S P S PO WT C WT O WT P WT PO
Ratio phospho to total p
1: swe/swe
2: WT C57Bl6

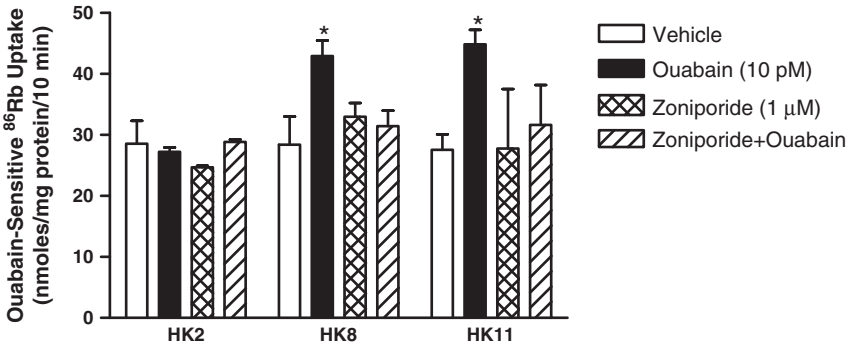


Fig. 4. Effect of NHE1 inhibition on ouabain-stimulated Na–K ATPase activity in renal proximal tubule cells. Three human kidney proximal tubule cell lines (HK2, HK8, or HK11) were treated for 15 min with ouabain (10 pM) in the presence or absence of zoniporide (1 μM). Ouabain (1 mM)-sensitive ^{86}Rb uptake was determined as a measure of Na–K ATPase-mediated ion transport. Each bar represents data (mean ± se) from 6 individual experiments (n = 6) performed in triplicate. *Indicates $P < 0.05$ as calculated by one-way ANOVA followed by Bonferroni analysis.

with digibind (Fig. 1B and C). Immunohistochemistry confirmed that the increase in Na–K ATPase α1 subunit (Fig. 1D) and NHE1 (Fig. 1E) expression in kidney tubules by ouabain was prevented by digibind.

3.2. Effect of NHE1 inhibition on ouabain-induced blood pressure

We have previously demonstrated that treatment with ouabain increases phosphorylation of NHE1, indicating that ouabain increases NHE1 activity [10]. To determine if NHE1 activity is required for the ouabain-mediated increase in blood pressure, animals were treated with ouabain in the presence or absence of zoniporide, a specific NHE1 inhibitor. As shown in Fig. 2A, zoniporide completely prevented the ouabain-stimulated increase in blood pressure. Zoniporide also blocked the ouabain-mediated increase in expression (Fig. 2B, D, & E) and activity (Fig. 2C) of Na–K ATPase α1 in renal basolateral membranes. Of note, zoniporide decreased basal expression of NHE1 in addition to blocking the ouabain-mediated effect on this protein (Fig. 2B, D & F).

3.3. Effect of NHE1 inhibition on ouabain-induced ^{86}Rb uptake and signaling pathway activation

Our prior studies, using a relatively nonspecific inhibitor of NHE1, had suggested that ouabain-mediated stimulation of Na–K ATPase activity and signal transduction pathway activation were dependent on NHE1 [10]. To directly test the requirement for NHE1 in ouabain signaling, we compared low-dose ouabain responses between NHE1-null cell lines with NHE1-expressing cell lines.

The NHE1-deficient proximal tubule cell lines used are the human HK2 cells and mouse SWE cells, while NHE1-replete cell lines used are the human HK11 cells and mouse WT cells. SWE cells are derived from NHE1-knockout mice and WT cells are derived from wild-type littermates of NHE1-knockout mice. To determine if a lack of NHE1 expression affects Na–K ATPase expression, we determined the expression of Na–K ATPase α1 and NHE1 in human and mouse kidney proximal tubule cell lines. As shown in Fig. 3A & B right-hand panels, a lack of NHE1 expression does not affect basal expression of Na–K ATPase α1 subunits. The cells were treated for 15 min with either 10 pM (human) or 100 nM (mouse) ouabain followed by western blot analysis of EGFR, Src, and ERK phosphorylation. As shown in Fig. 3, ouabain increased phosphorylation of EGFR, Src, and ERK in HK11 and WT cells. Pretreatment with the Src inhibitor PP2 prevented the increase in EGFR, Src, and ERK phosphorylation. However, neither ouabain nor PP2 had any effect on ouabain-stimulated EGFR, Src, and ERK phosphorylation in cells lacking NHE1 expression (HK2 and SWE). Interestingly, basal Src, EGFR, and ERK phosphorylation and total expression were significantly higher in both NHE1-deficient HK2 and SWE cells than NHE1-replete HK11 and WT mouse proximal tubule cells.

Pharmacological inhibition of NHE1 in the human proximal tubule cell lines by zoniporide (HK2, HK8, or HK11) blocked the ouabain-mediated increase in ^{86}Rb uptake in the NHE1-replete cells (HK8 and HK11). As expected, neither ouabain nor zoniporide had any effect on ^{86}Rb uptake in NHE1-null HK2 cells (Fig. 4). Of note, the basal Na–K ATPase-mediated ^{86}Rb uptake in NHE1-null cells (HK2 cells) was not significantly different from NHE1-expressing (HK8 or HK11) cells.

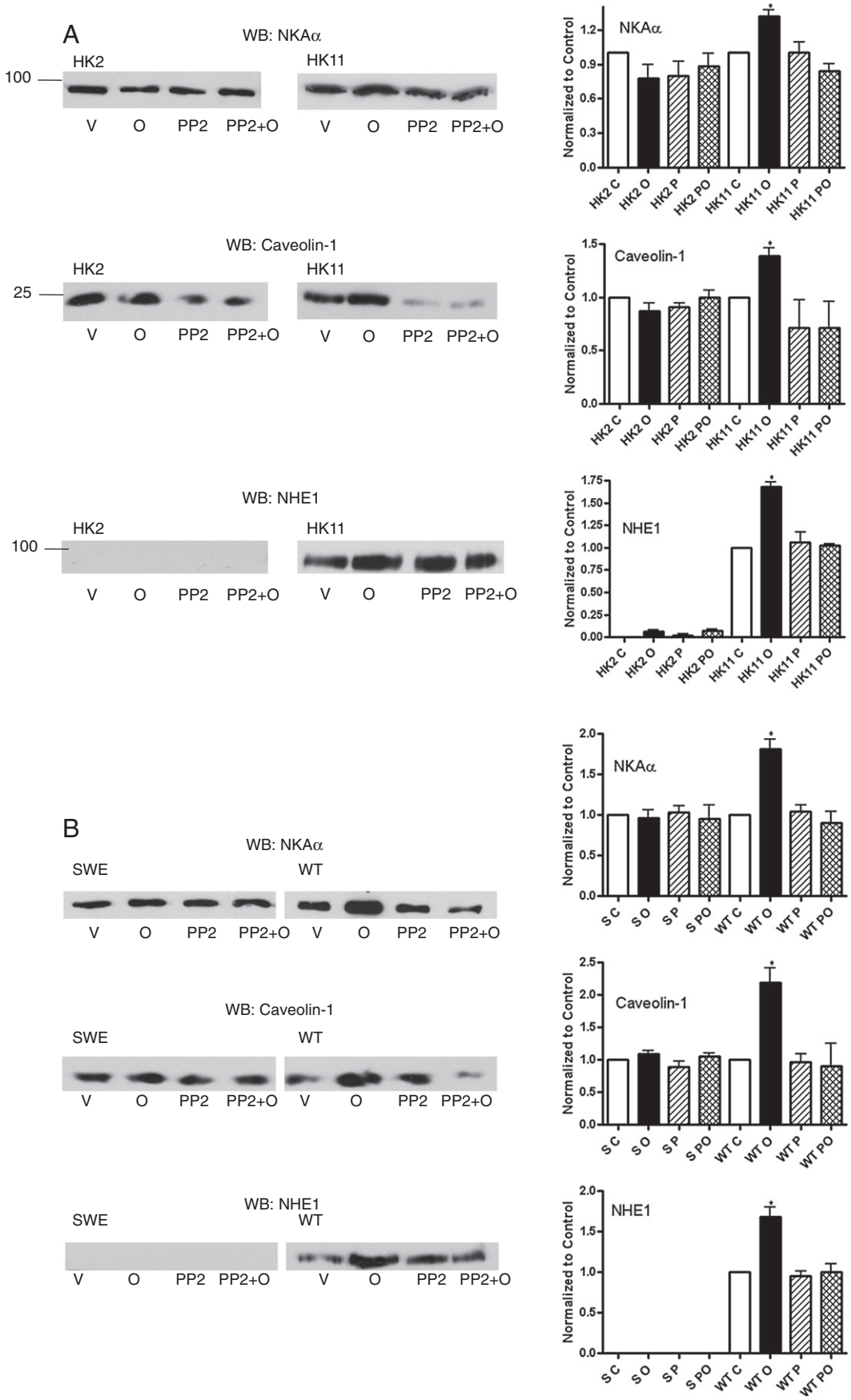
3.4. Requirement of NHE1 and Src phosphorylation in ouabain-stimulated Na–K ATPase α1 membrane expression

Our data support a requirement for NHE1 in mediating the effects of low-dose ouabain on Na–K ATPase function. To further validate the role of NHE1, we examined the relationship between ouabain-stimulated NHE1 expression, Na–K ATPase expression, and Src phosphorylation in the NHE1-null (HK2 and SWE) and -replete (HK11 and WT) cell lines. Cells were treated for 15 min with ouabain in the presence or absence of PP2. Cells were then subjected to surface biotinylation followed by precipitation of biotinylated proteins using streptavidin beads and analyzed by western blot for Na–K ATPase α1, caveolin-1, or NHE1. As shown in Fig. 5, ouabain increased the membrane expression of Na–K ATPase α1, caveolin-1, and NHE1 in HK11 and WT cells. Pretreatment with PP2 abolished the increase in membrane expression of all three proteins. However, neither ouabain nor PP2 had any effect on the expression of any of the proteins studied in the NHE1-null HK2 and SWE cells.

3.5. Association between Na–K ATPase α1 and NHE1

Using immunoprecipitation, we have previously demonstrated that treatment with low dose ouabain increases the association between Na–K ATPase α1 and NHE1 [10]. To determine the nature and localization of this association, NHE1-null SWE cells were transiently transfected with mCherry-tagged rat Na–K ATPase α1 and GFP-tagged human NHE1. FRET or TIRF microscopy was performed in live cells before and after treatment with 100 nM ouabain. As shown in Fig. 6A, epifluorescence showed that both proteins were expressed in the

Fig. 5. Effect of NHE1 and Src kinase on ouabain-stimulated Na–K ATPase α1 expression in kidney proximal tubule cells. Cells were treated for 15 min with 10 pM (A, HK2 and HK11 cells) or 100 nM (B, SWE and WT cells) ouabain in the presence or absence of PP2 (1 μM) and cell surface was biotinylated as described in Methods. Crude membrane proteins were separated by 10% SDS-PAGE and analyzed by immunoblot using Na–K ATPase α1 subunit (upper panel), caveolin-1 (middle panel), or NHE1 (lower panel) antibodies. Values (mean ± se) as fold difference from the vehicle-treated group represent densitometry data from three independent experiments as Arbitrary Units (AU). *Indicates $P < 0.05$ as calculated by two-way ANOVA followed by Bonferroni analysis.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HK2
HK11
100
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Normalized to Control
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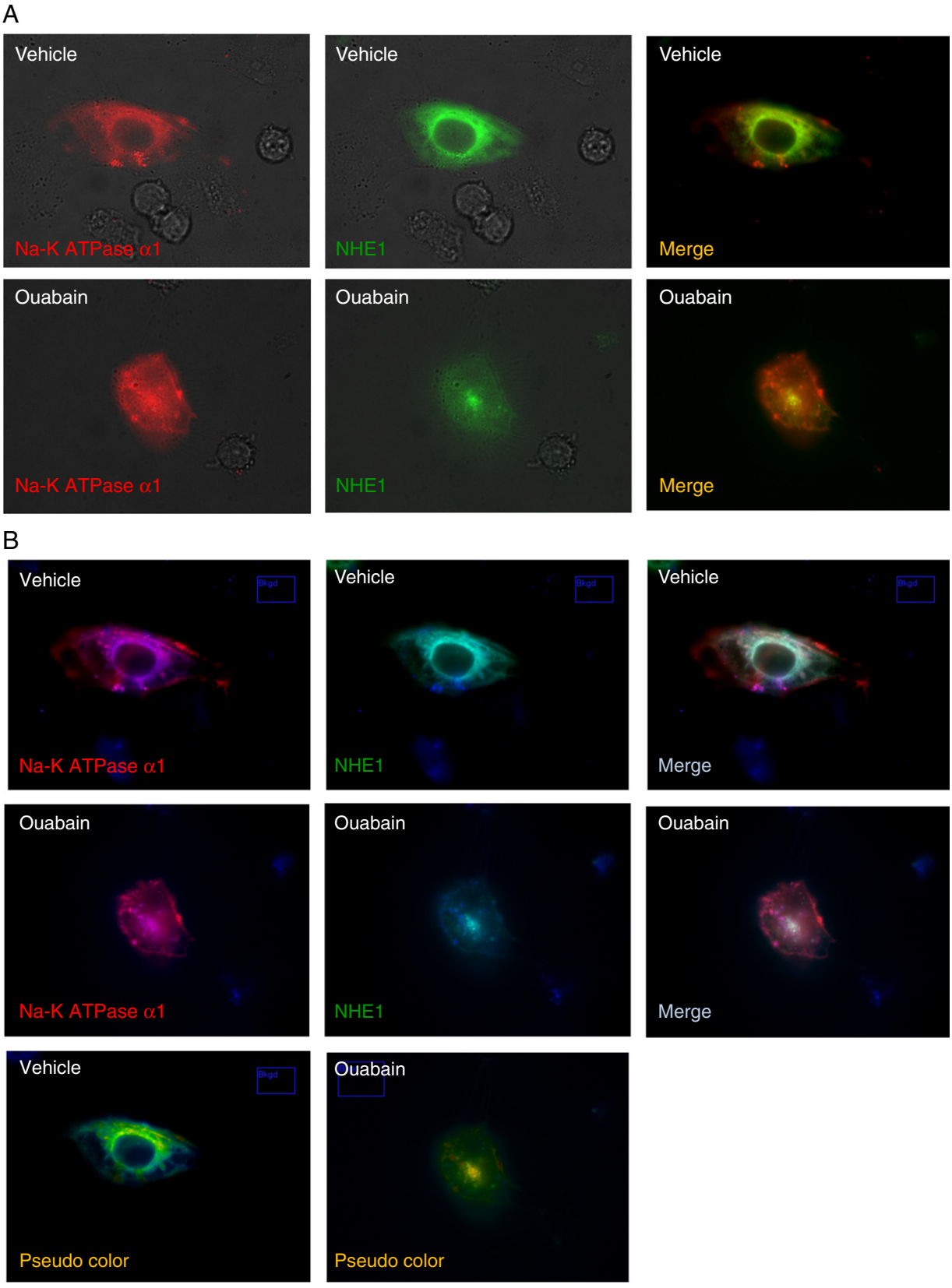

Fig. 6. Effect of ouabain on association between Na–K ATPase α1 and NHE1 in kidney proximal tubule cells. Immortalized proximal tubule cells from NHE1 knock-out mice (swe/swe) were transfected with mCherry-tagged rat Na–K ATPase α1 (red fluorescence) and/or GFP-tagged NHE1 (green fluorescence). After 24 h cells were imaged for epifluorescence (A), sensitized FRET (B), or TIRF (D) microscopy before and after treatment with ouabain (100 nM) as described in Methods. Representative images are shown. Sensitized FRET after photo bleaching was calculated using SlideBook4.2 software as described in Methods. (C) Each bar represents data (mean ± se) normalized to donor or acceptor emission from six individual experiments ($n = 6$). In each experiment 30–40 cells were imaged and data from all the cells were pooled and considered as one data point. *Indicates $P < 0.05$ as calculated by two-way ANOVA followed by Bonferroni analysis.

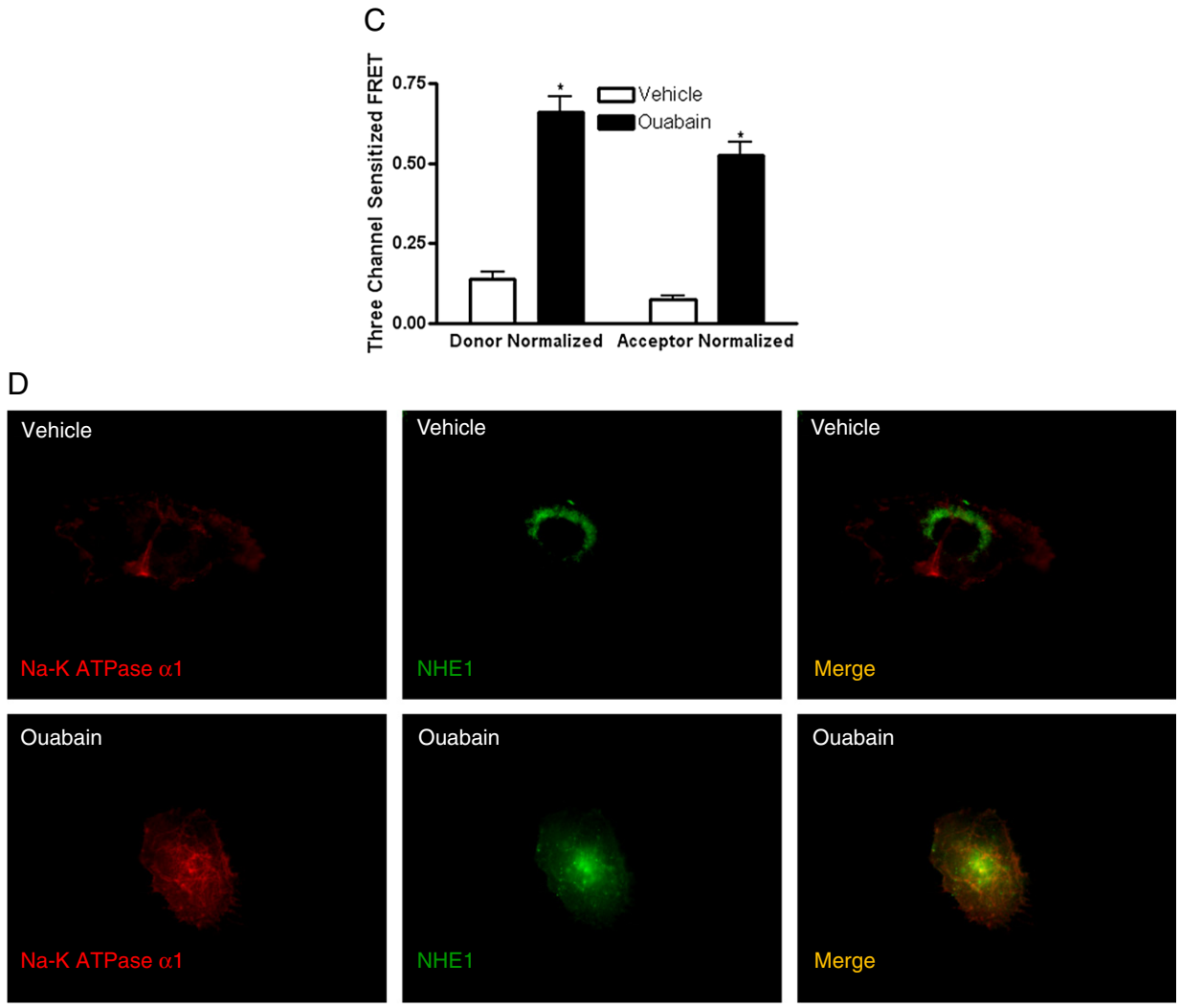

Fig. 6 (*continued*).

cells. FRET microscopy showed that the two proteins are in close proximity even under basal conditions, and that the interaction between the two increased after treatment with ouabain (Fig. 6B). Quantification of data showed that sensitized FRET efficiency after photobleaching increased significantly after ouabain treatment (Fig. 6C). Ouabain also stimulated a dramatic change in the pattern of protein expression as seen in Fig. 6A and D. To determine if ouabain increases basolateral membrane expression of Na–K ATPase α1 and NHE1, TIRF microscopy was performed in the same cells following FRET microscopy. As shown in Fig. 6D, treatment with ouabain increased expression and association between Na–K ATPase α1 and NHE1 in the membrane compartment.

3.6. Molecular domains of Na–K ATPase α1 and NHE1 involved in ouabain-stimulated association

To identify the molecular domains in Na–K ATPase α1 required for association with NHE1, YFP-tagged CBM mutant of Na–K ATPase α1 (pseudo color green) and full-length RFP-tagged NHE1 (red color) were transfected in SWE cells. Epifluorescence and TIRF microscopy were performed before and after treatment with 100 nM ouabain. As shown in Fig. 7A, epifluorescence showed that both Na–K ATPase α1 and NHE1 are expressed in transfected cells and the expression did not change after treatment with ouabain. However, TIRF microscopy showed that the CBM mutant of Na–K ATPase α1 was not expressed in the basolateral membrane. Treatment with ouabain failed to increase the membrane expression of Na–K ATPase α1. Interestingly, transfection of CBM-mutant Na–K ATPase α1 prevented the membrane expression of NHE1, suggesting that expression of the caveolin binding motif of the Na–K ATPase α1 subunit is critical for membrane expression of both Na–K ATPase α1 and NHE1 (Fig. 7B).

NHE1 expresses a distinct N-terminal activity domain and a C-terminal scaffolding domain which facilitate formation of signalosomes [18]. To determine if either or both the activity and scaffold domains of NHE1 are required for Na–K ATPase stimulation by low-dose ouabain, NHE1-deficient SWE cells were transiently transfected with HA-tagged full-length wild type, activity-domain-mutated (E266I), or scaffold-domain-mutated (KR/A) NHE1 constructs in pCMV vector. Expression was confirmed by western blot using anti-HA tag antibodies (data not shown). Transfected cells were treated for 15 min with 100 nM ouabain, and Na–K ATPase-mediated ^{86}Rb uptake was measured. As controls, WT (positive control), SWE (negative control), or SWE cells transfected with vector only (negative control) were also analyzed along with the cells transfected with NHE1. As shown in Fig. 7C, treatment with ouabain increased Na–K ATPase-mediated ^{86}Rb uptake in WT and SWE cells transfected with full length NHE1. Treatment with ouabain did not have any effect on ^{86}Rb uptake in SWE cells transfected with vector or activity-domain-mutated NHE1 (E266I) cells. Paradoxically, low-dose ouabain significantly decreased Na–K ATPase-mediated ^{86}Rb uptake in

SWE cells transfected with scaffold-domain-mutated NHE1 (KR/A). To determine whether mutations in NHE1 affect association with Na–K ATPase α1 subunit, crude membranes from transfected cells treated with vehicle or ouabain were prepared and immunoprecipitated using anti-HA antibodies, followed by western blot for the Na–K ATPase α1 subunit. As shown in Fig. 7D, ouabain increased the association between the Na–K ATPase α1 subunit and NHE1 in cells transfected with full length (WT) NHE1 and activity-deficient-mutant NHE1 (E266I), but not in scaffolding-mutant NHE1 (KR/A). Interestingly, the basal association between the Na–K ATPase α1 subunit and the KR/A mutant was higher than the association with either WT or E266I mutant.

4. Discussion

This study provides evidence that low-dose ouabain stimulates a direct interaction between the Na–K ATPase α1 subunit and NHE1,

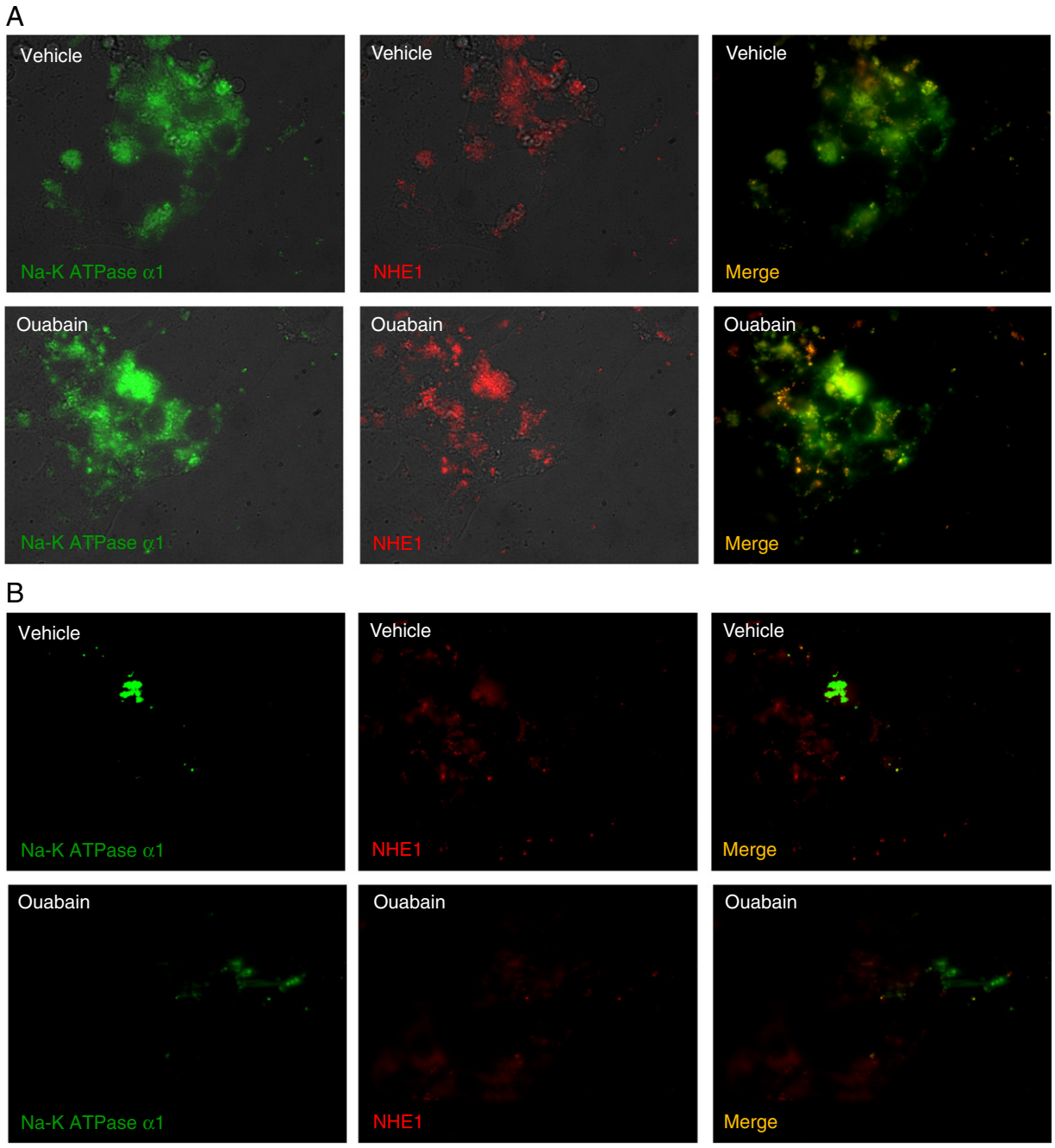

Fig. 7. Effect of mutations in Na–K ATPase α1 and NHE1 on ouabain-stimulated association between Na–K ATPase α1 and NHE1 in kidney proximal tubule cells. Immortalized proximal tubule cells from NHE1 knock-out mice (swe/swe) were transfected with YFP-tagged rat Na–K ATPase α1 with the mutation in caveolin binding motif (pseudo color green fluorescence) and/or RFP-tagged NHE1 (red fluorescence). After 24 h cells were imaged for epifluorescence (A) or TIRF (B) microscopy before and after treatment with ouabain (100 nM). Representative images are shown. (C) SWE cells were transiently transfected with full-length, activity-deficient (E266I) mutant or scaffold-deficient (KR/A) mutant NHE1. Cells were treated with 100 nM ouabain and Na–K ATPase-mediated ^{86}Rb uptake was measured as described in Methods. Each bar represents data (mean ± se) from 6 individual experiments (n = 6) performed in triplicate. *Indicates $P < 0.05$ by one way ANOVA followed by Bonferroni analysis. (D) SWE cells were transiently transfected with the indicated plasmids and treated with ouabain (100 nM). Crude membranes were prepared, resuspended in IP lysis buffer, and immunoprecipitated with anti-HA antibodies. Representative western blots (n = 2) using anti-Na–K ATPase α1 subunit or HA-tag are shown. A separate gel was run using a fraction of proteins used for immunoprecipitation and blotted against anti-actin antibodies to show equal amount of proteins used for immunoprecipitation.

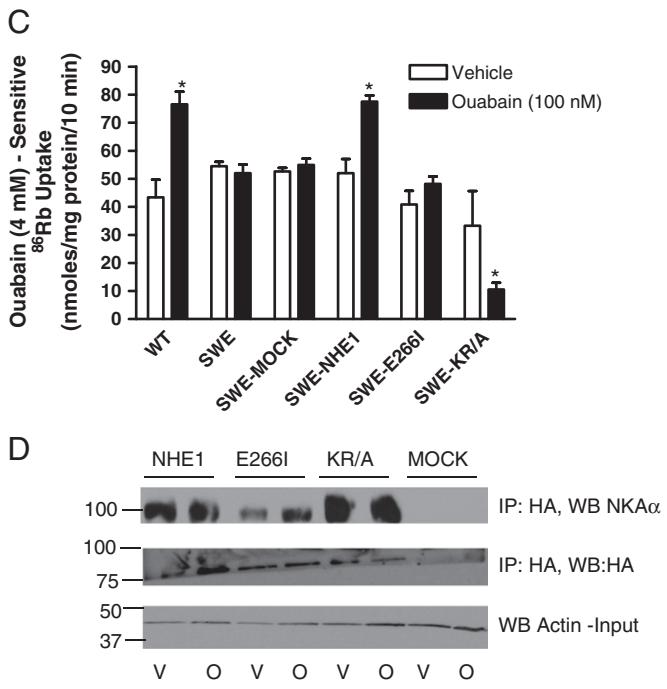

Fig. 7 (*continued*).

and that this interaction requires the caveolin binding motif of the α1 subunit as well as both the activity and scaffolding domains of NHE1. Using NHE1-deficient cell lines, we have directly shown a requirement for NHE1 for activation of the EGFR/Src/ERK/Akt pathway by low-dose ouabain. These findings have significant implications for understanding the regulation of proximal tubule transepithelial ion transport and sodium homeostasis.

The Na–K ATPase is the key regulator of sodium reabsorption in the proximal tubule, where greater than 60% of renal sodium reabsorption occurs [28]. Relatively small changes in transport at this site have the ability to effect major changes in ion homeostasis. In the renal proximal tubule, Na–K ATPase is heavily regulated by hormones [29], neurotransmitters [30,31], and cardiac glycosides [32,33]. While initially thought to have solely an inhibitory effect on the Na–K ATPase activity, cardiac glycosides are now recognized as instigators of a signaling pathway involved in the regulation of cell proliferation and survival [4,6,7,9,11]. The involvement of NHE1 in this function is fitting, in that NHE1 has been implicated in a variety of cell functions, including maintenance of intracellular pH, growth, and survival [12–18]. Interestingly, the concentrations of ouabain and some other cardiac glycosides that stimulate this signaling pathway are considerably lower than the concentrations that inhibit the pump. How the stimulatory effect of ouabain on the Na–K ATPase activity interfaces with the other well-known regulatory factors is not understood. Furthermore, whether all cardiac glycosides have a similar effect is not clear.

FRET analysis is consistent with a direct interaction between NHE1 and the α1 subunit of Na–K ATPase. Analysis of proteins with defined mutations implicates the caveolin binding motif of Na–K ATPase α1 and both the activity and scaffolding domains of NHE1 as crucial elements for ouabain-induced α1-NHE1 association. However, our data do not allow us to draw the conclusion that the caveolin binding motif of Na–K ATPase α1 interacts with specific sites within the activity and/or scaffolding domains of NHE1. The failure of the caveolin-binding-motif-mutated Na–K ATPase α1 subunit to show normal expression at the basolateral membrane suggests that one reason for the lack of interaction between the mutated Na–K ATPase α1 and NHE1 is simply abnormal localization of Na–K ATPase α1. Likewise, the failure of ouabain to stimulate Na–K ATPase activity with NHE1 that lacks a functional activity domain may suggest that the interaction between Na–K ATPase α1 and NHE1 occurs in that domain; however, another potential explanation is that ouabain-stimulated NHE1 activity facilitates interaction at another site. The effect of the scaffolding domain NHE1 mutant on the response to ouabain is of some interest. Absence of a functional scaffolding domain abolished the stimulatory effect of ouabain on Na–K ATPase activity and uncovered an inhibitory effect of low-dose ouabain on Na–K ATPase activity, suggesting that the scaffolding domain of NHE1 may regulate the sensitivity of Na–K ATPase to ouabain. The increased association between Na–K ATPase and NHE1 with KR/A mutation would suggest that the association probably occurs at a site other than the putative scaffolding domain of NHE1. Further studies are required to confirm this conclusion.

The investigations into the signaling pathway suggest other roles for NHE1 vis a vis regulation of Na–K ATPase function. The phosphorylation status and total expression of EGFR, Src, and ERK in the NHE1-null HK2 and SWE cells were significantly higher under basal conditions than HK11 and wild type mouse proximal tubule cells. In contrast to HK11 and wild type mouse proximal tubule cells, the NHE1-deficient cell types (HK2 and SWE) showed no increase in phosphorylation of these signaling compounds in response to ouabain treatment. These data may suggest that total ablation of NHE1 expression may evoke compensatory changes in regulatory pathways. This is probably not unexpected given its likely role in adjusting ionic homeostasis and cell proliferation. These data may also suggest that the NHE1/Na–K ATPase complex sequesters Src under basal conditions but upon treatment may release activated Src to phosphorylate EGFR and downstream signaling pathways. However, further studies are required to confirm this conclusion.

Our studies suggest that the increase in ^{86}Rb uptake by low dose ouabain is accompanied with an increase in membrane expression of Na–K ATPase. The increase in expression of Na–K ATPase required NHE1 expression and activity as inhibition of NHE1 by zoniporide prevented the increase in Na–K ATPase membrane expression. Cell culture studies suggest that the increase in expression was dependent upon Src kinase. We have previously demonstrated that treatment with low dose ouabain increases phosphorylation of the Na–K ATPase α1 subunit and NHE1 [10]. Taken together, the data suggest that phosphorylation of Na–K ATPase and NHE1 through Src dependent mechanisms increases translocation of Na–K ATPase and NHE1. Further studies using mutations at specific sites are required to confirm this conclusion.

In summary we have described an ouabain-stimulated direct interaction between Na–K ATPase α1 subunit and NHE1 which is critical for ouabain-stimulated Na–K ATPase activity and signal transduction. The regulatory role for NHE1 in these processes suggests potential therapeutic targets for regulation of both proximal tubule sodium transport and cell survival and proliferation.

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