The calmodulin antagonist W-7 inhibits the epithelial Na+/H+ exchanger via modulating membrane surface potential

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HE3 is regulated via alterations in membrane surface charge. This is achieved through altered binding of cationic regions in the cytosolic-terminus of the exchanger with the inner leaflet of the plasma membrane. Calmodulin antagonists, including W-7, regulate surface potential and inhibit NHE3 activity. Utilizing fluorescent protein conjugated membrane probes we show that binding of cationic, but not hydrophobic peptides, to the plasma membrane is prevented by W-7. An interaction between cationic regions in the regulatory, cytosolic domain of NHE3 to anionic phospholipids in either reconstituted liposomes or the plasma membrane in cell culture is similarly prevented by W-7, at a concentration that inhibits the exchanger. We propose therefore that W-7 inhibits NHE3 activity, at least in part, by altering the association of cationic segments within the carboxy-terminus of the exchanger with anionic phospholipids in the plasma membrane.

Introduction

The sodium proton exchangers (NHEs) are a diverse family or proteins that share a common structure and mediate the electroneutral exchange of a single sodium ion for a proton. The third isoform, NHE3, is predominately expressed in the apical membrane of renal and intestinal epithelia. Consequently NHE3 has been called the epithelial sodium proton exchanger. It contributes fundamentally to the maintenance of intravascular volume and pH. Given the myriad of perturbations to volume and pH homeostasis incurred by an organism, minute-to-minute adjustments of exchanger activity are necessary. This acute regulation requires the cytosolic regulatory domain of NHE3 and is conferred via a multitude of signaling pathways and second messengers, not the least of which is altered intracellular levels of calcium (Ca2+).2 Interestingly, depending on the model system employed increased cytosolic Ca2+ either inhibits NHE3 activity2,3 or stimulates the exchanger.7,10 The mechanisms(s) mediating these effects are incompletely delineated. Regulation of NHE3 by altered cytosolic Ca2+ has been reported to occur via: the activation of PLC, formation of multi-protein complexes (in particular complexes containing the NHERF’s), direct effects of free cytosolic Ca2+ itself and/or via the activation of protein kinases including calmodulin/calcmodulin kinase (reviewed in ref. 1).

The calmodulin antagonists, including W-7, inhibit NHE3 activity4,12 and prevent Ca2+ mediated activation of the exchanger.7,9 This inhibition is not additive with inhibition of NHE3 activity induced by hyperosmolarity,11 inferring that they may share a common inhibitory mechanism. It is assumed that the effect of W-7 and other membrane permeant calmodulin inhibitors was to prevent the association of NHE3 with calmodulin or the activation of calmodulin kinase. However, recent evidence suggests that the membrane-permeant calmodulin inhibitors mediate their effects, at least in part, via binding to anionic phospholipids in the inner leaflet of the plasma membrane and shielding its negative surface charge.13
Interestingly, we recently demonstrated that membrane surface charge alters both the structure and function of the epithelial sodium proton exchanger. This is conferred via a regulated electrostatic interaction between cationic regions within the cytosolic terminus of the exchanger with anionic phospholipids in the inner leaflet of the plasmalemma. These studies revealed that both elevated cytosolic Ca$^{2+}$ and hyperosmolality could prevent this interaction and dislodge the tail of the exchanger from the plasma membrane, thereby inhibiting exchanger activity.

Given the recent evidence that the membrane permeant calmodulin inhibitors alter membrane surface potential and our observation that membrane potential dictates NHE3 activity we hypothesized that W-7 prevents the binding of the cationic regions in the regulatory domain of the exchanger to the plasma membrane, thereby inhibiting NHE3 activity. To this end we demonstrate here that W-7 prevents the binding of cationic segments in the cytosolic terminus of NHE3 to liposomes composed of anionic phospholipids, dislodges these regions from the plasma membrane and inhibits exchanger activity.

**Results**

Given the recent evidence that W-7 can bind anionic phospholipids in vitro, we first determined the effect of W-7 on membrane surface potential in live cells. To do so we employed two well-characterized probes of membrane surface properties: R-pre and Palm. Both probes are conjugated to RFP and associate with the plasma membrane when transfected into live cells (Fig. 1A and C). However, the mechanism mediating their membrane localization is very different. R-Pre is composed of nine arginines followed by a single farnesylation motif. Consequently, it associates with the inner leaflet of the plasma membrane via an electrostatic interaction. Consistent with this, perturbations that alter surface charge dislodge this probe from the membrane.

Alternatively, Palm is composed of a single farnesyl and di-palmitoylation motifs. It associates with membranes exclusively via a hydrophobic interaction. Importantly, its membrane localization is not altered by cellular perturbations that eliminate surface charge. Each probe was transfected independently into OK cells and their membrane localization followed after incubation with 100 μM W-7 (a concentration sufficient to affect NHE3 activity and Fig. 4). W-7 displaced R-Pre from the membrane within 20 minutes of its addition, however Palm remained adhered to the plasma membrane (Fig. 1B and D). This demonstrates that W-7 significantly alters the surface potential of the plasma membrane in situ, without affecting its integrity.

Next we examined the effect of W-7 on the binding of the cationic regions within the C-terminus of NHE3 to lipids in vitro. For these studies we synthesized peptides corresponding to each of the three cationic regions within the cytosolic C-terminus of NHE3 and conjugated bimane to the N-terminus. Bimane is a fluorescent reporter whose intensity increases when closely associated with a lipid bilayer. For simplicity hereafter we refer to each segment as: region 1 (residues 456–480), region 2 (residues 503–527) and region 3 (residues 673–688). Previously, it was shown that these peptides preferentially adhere to anionic rather than zwitterionic liposomes. This interaction is electrostatic in nature as the addition of cationic sterols or an increase in ionic strength prevents the association of each region with anionic liposomes. For all three bimane conjugated peptides incubated with a fixed concentration of anionic liposomes (20 mole % phosphatidyl serine, a composition which approximates that of the plasma membrane) the addition of W-7 quenched fluorescence (Fig. 2). This is consistent with W-7 abolishing the electrostatic interaction between these peptides and anionic liposomes. In contrast, the addition of W-7 to solutions containing the peptides and
W-7 displaces cationic peptides corresponding to regions within the cytosolic domain of NHE3 from anionic liposomes. Relative fluorescence of bimane conjugated peptides corresponding to cationic Regions 1, 2 and 3’ within the cytosolic domain of NHE3, in solution with either liposomes composed of PS or PE (20% mol fraction) and an increasing concentration of W-7. Each point in the graph represents the mean value of at least three independent experiments per condition.
plasma membrane reorients itself in such a way that it obstructs the pore, thereby preventing ion flux. One could envision such a mechanism existing for NHE3. Alternatively disruption of an electrostatic interaction between the cytosolic domain of NHE3 and the plasma membrane may alter the association of the exchanger with multi-protein complexes and or with the actin cytoskeleton. This may in turn permit altered sub-cellular localization of the exchanger. However, while decreased surface expression of the exchanger would decrease activity, mutation of the cationic regions does not alter surface expression of NHE3.14

Elevated intracellular Ca\textsuperscript{2+} regulates NHE3 activity.1 The proposed mechanism mediating this includes: activation of protein kinases, altered cellular localization and the formation of multi-protein complexes. NHE3 is held fixed in its functional domain, the apical plasma membrane, via a link to the actin cytoskeleton.18,19 The molecular tether mediating the association of NHE3 with the actin cytoskeleton has been reported to be via NHERF and ezrin or directly through ezrin.20,21 NHERF family members participate in Ca\textsuperscript{2+} mediated alterations of NHE3 activity via regulating the association of the exchanger with the actin cytoskeleton/regulatory complexes with and without altering cell surface expression.21 Moreover, some of the NHERFs are necessary for basal exchanger activity, yet do not affect the amount of NHE3 in the plasma membrane.22 This infers that NHE3 in the plasma membrane, which is not bound to actin, is less active and that altering an association with the actin cytoskeleton may alter the intrinsic activity of NHE3. Interestingly, ezrin binds to the second cationic region within the tail of NHE3 that we show here binds to the inner leaflet of the plasma membrane and whose interaction is disrupted by W-7.20 Alterations in expression of ezrin affect NHE3 activity.23 Moreover, ezrin can bind the anionic phospholipid, PIP\textsubscript{2}, a process that prevents self-binding, permitting it to function as a linker.

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**Figure 3.** W-7 prevents the association of the cationic regions within the cytosolic domain of NHE3 with the plasma membrane. Representative confocal images of GFP conjugated Regions 1, 2 and 3’ of NHE3 cytosolic domain before (A, C and E) and 20 minutes after (B, D and E), their incubation with 100 μM W-7. The images above the main parts are X-Z reconstructions. Images presented are representative of the effect observed from at least three independent experiments examining greater than 12 cells in total per condition.
molecule between membrane proteins and the actin cytoskeleton. Further, the region of NHE3 that binds ezrin also interacts directly with phosphoinositides, including PIP2. Perhaps, disruption of membrane surface charge also disrupts an interaction between NHE3 and ezrin, severing a link between the exchanger and the actin cytoskeleton. As with some NHERFs, this may inhibit NHE3 activity without altering cell surface expression.

The concentration of W-7 necessary to inhibit Na+/H+ exchange (IC50 = 31 μM) is in the range of the inhibitory concentration for calmodulin (IC50 = 31 μM) as well as other targets; including myosin light chain kinase (IC50 = 51 μM) and protein kinase C (IC50 = 260 μM). Although effects have been observed at much smaller concentrations, for the majority of these studies, we choose to use a concentration of 100 μM, as this is the approximate concentration previously employed when investigating the effects of W-7 on NHE3 activity (75–150 μM). Although these and other findings are consistent with calmodulin antagonists mediating their effect on NHE3 via altering surface charge, this study does not definitively exclude a role for calmodulin and or the calmodulin kinases. Further, while it is likely that these effects are universal for all membrane permeant calmodulin inhibitors, it is important to note that our observations are limited to one, W-7.

In summary, W-7 prevents the association of cationic but not hydrophobic peptides with the plasma membrane. More specifically, W-7 prevents the binding of cationic regions within the cytosolic terminus of NHE3 to anionic phospholipids in liposomes and within the inner leaflet of the plasma membrane. Prevention of this interaction inhibits the exchanger. We therefore suggest that W-7 mediates inhibition of NHE3, at least in part, by preventing an association between cationic regions within the C-terminus of the exchanger and the inner leaflet of the plasma membrane.

**Materials and Methods**

**Cell culture, transfection and live cell imaging.** Opossum kidney cells (OK) were obtained from ATCC and were maintained in DMEM/F12 with 5% fetal bovine serum and antibiotics (penicillin and streptomycin) at 37°C in 5% CO2. For experimental purposes they were plated on 25 mm glass coverslips. During live cell imaging they were incubated with isotonic sodium buffer (140 mM NaCl, 3 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 20 mM HEPES pH 7.4 and 5 mM glucose). Transient transfection of RFP-R-Pre, Palm-RFP, GFP-(456–480)-farnesyl, myr-(503–527)-GFP and GFP-(673–688)-farnesyl was performed with FuGene6 (Roche) as per the manufacturers protocol using a ratio of 3 μg of plasmid to 5 μL of FuGene6. The details of plasmid construction can be found in references 14 and 15. Fluorescent images were obtained with a spinning-disk confocal microscope (WaveFx from Quorum Technologies, Guelph, Canada) set up on an Olympus IX-81 inverted stand (Olympus, Markham, Canada), employing a 60x objective. The system is equipped with diode-pumped solid-state laser lines (405, 440, 491, 561 and 638 nm; Spectral Applied Research, Richmond Hill, ON Canada), a motorized XY stage and a piezo focus drive. Images were obtained with an EMCCD camera (Hamamatsu, Japan) driven by velocity 5.0.3 software.

**Liposome binding assays with synthetic peptides.** Synthetic peptides were synthesized by Biosynthesis Inc., (Texas) and the method utilized to perform binding assays to liposomes was based on the one described by Yeung et al. Liposomes were prepared by dispensing the required amount of lipids (synthetic DOPC, POPs and POPE, all from Avanti) into a glass tube, after which the solvent, chloroform, was evaporated off under nitrogen. The dry lipids were then re-suspended in 20 mM Tris-HCl, pH 7.2, before they were passed through a liposome extruder (Avestin, Ottawa) containing a 100-nm pore-size polycarbonate filter. This produced large unilamellar vesicles. Liposomes were predominantly made up of DOPC with a smaller fraction of other lipids (20 mol% of either PE or PS). As the peptides were conjugated to bimane, their approximation to liposomes was assessed by fluorescence measurements (excitation 390 nm, emission 468 nm). Note that bimane fluorescence is altered.

![Figure 4.](image-url)
in a hypophosphoric environment. For these studies a constant amount of bimane-conjugated peptide (-1 nmol) was added to a glass cuvette (Starna Cells Inc., California) containing a fixed amount of liposomes suspended in 20 mM Tris-HCl buffer. The indicated concentrations of W-7 (Calbiochem) were added and then fluorescence was measured with a Hitachi spectrophotometer F-2500. For each series of experiments, background fluorescence from the buffer alone was subtracted from all readings. The measured fluorescence intensities were normalized to the intensity observed from the peptide incubated in the absence of liposomes, this value was arbitrarily set to 1.

**Measurement of NHE3 activity.** The activity of NHE3 was assessed by measuring the rate of pH recovery, induced by the presence Na⁺, after an acid load. We used dual excitation ratio measurements of BCECF to measure pH, as detailed previously in reference 18. Cells were grown on 25 mm glass coverslips, placed into Attotefluor cell chambers and mounted on the stage of the microscope. They were then loaded with 5 μg/ml BCECF-AM in Na⁺ buffer (details described above) and pre-pulsed with 50 mM NH₄Cl at 37°C for 10 min for acid loading. Extracellular dye and NH₄Cl were then washed away with Na⁺-free solution and Na⁺/H⁺ exchange initiated by adding back the Na⁺-containing solution. Cytosolic pH was calibrated by equilibrating the cells with K⁺-rich media titrated to specific pH values in the presence of 10 μg/ml nigericin, immediately after the recovery of pH for each experimental condition.

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