RESEARCH ARTICLE

ENaC activity is regulated by calpain-2 proteolysis of MARCKS proteins

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1Department of Physiology and Functional Genomics and Department of Medicine Division of Nephrology, Hypertension, and Renal Transplantation, University of Florida College of Medicine, Gainesville, Florida; 2College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing, China; and 3Department of Physiology, Emory University School of Medicine, Atlanta, Georgia

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Montgomery DS, Yu L, Ghazi ZM, Thai TL, Al-Khalili O, Ma HP, Eaton DC, Alli AA. ENaC activity is regulated by calpain-2 proteolysis of MARCKS proteins. Am J Physiol Cell Physiol 313: C42–C53, 2017. First published May 3, 2017; doi:10.1152/ajpcell.00244.2016.—We previously demonstrated a role for the myristoylated alanine-rich C kinase substrate (MARCKS) in regulating ENaC and ENaC in cultured renal epithelia and in the mouse kidney. Using recombinant fusion proteins, we showed that MARCKS and ENaC are regulated by proteolysis; Calpains are a family of ubiquitously expressed intracellular Ca2+-dependent cysteine proteases involved in signal transduction. Here we examine the role of calpain-2 in regulating MARCKS and ENaC in cultured renal cells. Ca2+-mobilization and calpain-2 inhibition decrease the association between ENaC and MARCKS. The inhibition of calpain-2 reduces ENaC activity as demonstrated by single-channel patch-clamp recordings and transepithelial current measurements. These results suggest that calpain-2 proteolysis of MARCKS promotes its interaction with lipids and ENaC at the plasma membrane to allow for the phosphatidylinositol 4,5-bisphosphate (PIP2) (31). The ability of members of the MARCKS family of proteins to function as PIP2-sequencing proteins at the membrane is dependent on hydrophobic interactions between the myristoylation domain and the plasma membrane and electrostatic forces between the effector domain and the plasma membrane (40).

Proteolytic cleavage activates ENaC by removing inhibitory peptides and producing conformational changes of the protein (28). Several different proteases including furin (25), prostasin (9), elastase (10), plasmin (35), kallikrein (36), and cathepsins (6, 23) have been shown to regulate the activity of ENaC. Like ENaC, the function of MARCKS is also regulated by proteolysis.

Calpains are Ca2+-dependent cysteine proteases that play a key role in signal transduction. The two main calpain isoforms are µ-calpain and m-calpain; “µ” and “mc” correspond to calpain isoforms with micromolar and millimolar requirements for Ca2+, respectively (11, 15). The Ca2+ concentrations required for half-maximal activity of calpains are several orders of magnitude greater than normal resting Ca2+ concentration within the cell (20). Despite this, calpains are demonstrably activated within cells presumably by high local concentrations of calcium or by increasing the sensitivity of calpains to cofactors such as phosphatidylinositol phosphates. There are at least 15 different calpains (34). All of these calpains are heterodimers consisting of a catalytic subunit and a regulatory subunit (41), and it has been suggested that these proteases are activated by autoproteolysis (32). All of our work was done with the ubiquitously expressed calpain-2. The physiological roles of calpains are widespread and diverse, including remodeling of the cytoskeleton and Ca2+-dependent signal transduction (14). Unlike some enzymes that are involved in protein degradation, calpains make highly specific, limited cuts in proteins to modulate their function (11). For example, calpain has been shown to be an endogenous regulator of MARCKS in airway epithelial cells (29). The ability of MARCKS to function as a scaffolding protein is dependent on its association with the cytoplasmic face of the plasma membrane. Calpain inhibition was reported to reduce the basal phosphorylation of MARCKS and its accumulation at the membrane of myoblasts (17) and rat hippocampal slices (16).
MARCKS plays an essential role in the PIP2-dependent regulation of ENaC gating in renal epithelial cells. The role of calpains to alter the function of either MARCKS or ENaC proteins in kidney cells has not been investigated. Taken together, the results from this study demonstrate that calpain-2 cleaves MARCKS but not ENaC subunits in kidney cells and calpain-2 proteolysis of MARCKS promotes its interaction with specific anionic lipid phosphates and its association with ENaC. These results also show that basal calpain-2 activity maintains MARCKS at the plasma membrane and ENaC in an open conformation. The modulation of calpain-2 activity in renal epithelial cells represents a novel mechanism for regulating the gating of ENaC by MARCKS in the kidney.

MATERIALS AND METHODS

Cell culture. Xenopus 2F3 cells (originally obtained from the late Dr. Dale Benos) were maintained in 4% CO2 at 26°C in Dulbecco’s modified Eagle’s medium (DMEM/Ham’s F-12 medium (Gibco, Grand Island, NY), pH 7.4 supplemented with 1.5 μM aldosterone (Sigma-Aldrich), 5% fetal bovine serum (FBS; Gibco), 1% streptomycin, and 0.6% penicillin (Gibco). Cells were plated on 12- and 24-mm Transwell permeable supports (Corning), and only cells from passages 99–106 were used for experiments. Mouse mpkCCDC14 cells were cultured in a 1:1 ratio of DMEM/Ham’s F-12 medium (Invitrogen) and supplemented with 2 mM l-glutamine, 1 nM triiodothyronine, 50 nM dexamethasone, 20 mM HEPES, 2% FBS, and 0.1% penicillin-streptomycin.

Reagents. Amiloride, the general calpain-2 inhibitor A6060 (19), the endogenous calpain inhibitor acetyl-calpastatin (SCP0063) (43), and the Ca2+ ionophore A23187 were purchased from Sigma-Aldrich.

Osmotic minipump infusion. SV129 wild-type mice were kept on a 12:12-h light-dark cycle and given standard chow with water ad libitum. All animal studies were approved and conducted in accordance with the policies of the Emory Institutional Animal Care and Use Committee. Osmotic minipumps (Alzet model 2002; Alzet Osmotic Pumps, Cupertino, CA) were filled with (20 μM) calpain-2 inhibitor A6060 prepared in DMSO. SV129 mice ~22 g in weight were anesthetized with ketamine-xylazine (129.22:7 mg/kg ip) before subcutaneously implanting the osmotic minipumps containing the calpain-2 inhibitor or 50% DMSO in sterile saline of the back of the mice. The calpain-2 inhibitor or 50% DMSO in sterile saline was infused at a rate of 0.5 μl/h for 7 days before the mice were euthanized and the kidneys were harvested for protein.

Recombinant protein expression and purification. Recombinant glutathione S-transferase (GST)-ENaCα, GST-ENaCβ, and GST-ENaCγ fusion proteins were expressed and purified as previously described (5, 6). The purity of each fusion protein was validated by mass spectrometry sequence analysis (Proteomics Core Facility at the Moffitt Cancer Center, Tampa, FL).

In vitro calpain-2 proteolytic assay. Calpain-2 purified from porcine kidney was purchased from EMD Millipore/Calbiochem (Billerica, MA). Purified recombinant GST-MARCKS (1 mg/ml) expressed and purified as previously described (3, 5, 6) was incubated with or without calpain-2 (1 mg/ml) in the presence of CaCl2 (final concentration, 5 mM) at 37°C for 1 h. The reaction was stopped by the addition of Laemmli buffer containing 5% β-mercaptoethanol and heating the sample at 95°C for 5 min. The sample was resolved and analyzed by SDS-PAGE.

Transepithelial current measurements. Mouse mpkCCDC14 cells were maintained in culture for 10 days before measuring the effect of various pharmacological inhibitors. After application of A6060 (a pharmacological inhibitor of calpain-2) or Ca2+ ionophore A23187, transepithelial voltages and transepithelial resistances across cell monolayers were recorded using an epithelial voltomhmer equipped with chopstick electrodes (World Precision Instruments, Sarasota, FL). At the end of the experiment, 100 nM amiloride was applied to the apical side of the Transwell permeable support. Amiloride-sensitive transepithelial current was calculated from Ohm’s law (Ie = Vd/Re, where Ie is transepithelial current, Vd is transepithelial voltage, and Re is transepithelial resistance) while correcting for the surface area of the insert.

Single-channel patch-clamp recordings. Patch pipettes were fabricated from filamented borosilicate glass capillaries (TW-150F; World Precision Instruments) with a two-stage vertical puller (PP-2; Narishige, Tokyo, Japan) with a resistance of 6–10 MΩ. Xenopus 2F3 cells were cultured on 12-mm permeable Transwell supports (Corning) or glutaraldehyde-sterilized, collagen-coated Millipore-CM filters (Millipore) that were mounted on the bottom of Lucite rings for patch-clamp experiments. Cells were visualized with Hoffman modulation optics (Modulation Optics) on a Nikon Diaphot. Negative pressure was applied to achieve a cell-attached patch with a seal resistance of 10–20 GΩ after making contact between the pipette tip and the cell surface. The extracellular bath and patch pipette solutions consisted of a saline solution with a pH of 7.3–7.4 and 95 mM NaCl, 3.4 mM KCl, 0.8 mM CaCl2, 0.8 mM MgCl2, and 10 mM HEPES. The cell-attached patch configuration was used for all single-channel experiments, and voltages are given as the negative of the patch pipette potential. ENaC activity within a patch was calculated using pCLAMP 10 software (Molecular Devices) as a product of the number of functional channels and the open probability.

SDS-PAGE and Western blot analysis. Mouse mpkCCDC14 cells were treated with the calpain inhibitor calpastatin or the calpain-2 inhibitor A6060 for 1 h at 37°C. Cellular lysates were prepared by washing the cell monolayer with 1X phosphate-buffered saline (PBS) and then scraping the cells in mammalian protein extraction reagent (MPER; Thermo Fisher Scientific, Rockford, IL) supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific). The cell lysate was passed through a 23-gauge syringe 10 times and then incubated on ice for 1 h. Tissue lysates were prepared by washing 50-mg pieces of tissue with 1X PBS followed by homogenization of the tissue in tissue protein extraction reagent (TPER; Thermo Fisher Scientific) supplemented with protease and phosphatase inhibitors (Halt; Thermo Fisher Scientific) while on ice and using an Omni TH homogenizer (Warrenton, VA). The tissue lysates were subject to centrifugation at 13,000 rpm at 4°C for 20 min, and the supernatant was sonicated twice for 10-s intervals while on ice. Bischloronic acid protein assay (Thermo Fisher Scientific) was used to determine protein concentration. One hundred micrograms of total protein from cellular or tissue lysates were loaded onto 4–20% Tris-HCl polyacryl- amide gels using the Criterion electrophoresis system (Bio-Rad). The resolved proteins were electrically transferred onto nitrocellulose membranes (GE Healthcare, Piscataway, NJ) using the Criterion transfer system (Bio-Rad). Membranes were blocked in 5% wt/vol milk in 1X Tris-buffered saline (TBS; Bio-Rad) at room temperature for 1 h, washed once with 1X TBS, and then incubated with primary antibodies ([ENaCα 58/59 (3)] or MARCKS (Abcam) at a dilution of 1:1,000 in 5% wt/vol BSA in 1X TBS while on a rocker at 4°C for 6–8 h. Membranes were washed three times with 1X TBS for 4-min intervals and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at a dilution of 1:3,000 prepared in blocking solution while on a shaker at room temperature for 1 h. Membranes were washed four times with 1X TBS for 4-min intervals, incubated with SuperSignal Dura Chemiluminescent Substrate for 5 min, and then imaged on a Kodak Gel Logic 2200 Imager as 16-bit grayscale images. Some images were normalized to loading controls (as noted), but all others were analyzed as raw pixel data using molecular imaging software (Carestream Health, Rochester, NY) or ImageJ (Fiji) using the gel analysis software.

PIP strip overlay binding assay. Nitrocellulose membranes spotted with 15 different phospholipids (Molecular Probes) were blocked with 3% fatty acid-free BSA in TBS-Tween 20 (TBS-T) at room temperature for 1 h. Membranes were incubated with 1 μg/ml of the GST
fusion protein or active calpain-2 in blocking buffer at 4°C for 8 h. Membranes were washed three times with TBS-T for 10-min intervals before being incubated with peroxidase-conjugated anti-GST antibody or anti-calpain-2 rabbit polyclonal antibody at 4°C for 4 h. Membranes were washed four times with TBS-T for 5-min intervals and then incubated directly with chemiluminescent substrates or with goat-anti rabbit secondary antibody and then chemiluminescent substrates before imaging the blots as previously described. To quantify the binding, the strips were imaged as 8-bit grayscale images. Using ImageJ, a circle was drawn around the darkest spot and the mean value of the pixels within the circle was measured. The same size circle was then used to measure the mean pixel density inside other spots on the image. This yields values between 0 (white) and 256 (black).

Sucrose density gradient assays. Mouse mpkCCDc14 cells were treated for 1 h with calpain-2 inhibitor A6060 and then scraped and

Fig. 1. SDS-PAGE and Coomassie staining analysis of MARCKS cleavage by calpain-2. A: recombinant GST and GST-MARCKS expressed and purified from BL21 cells are shown to have an electrophoretic mobility of 26 and 100 kDa, respectively. Active calpain-2 purified from porcine kidney is shown to have an electrophoretic mobility of 70 kDa in the presence of CaCl2. B: representative Coomassie gel showing the cleavage of GST-MARCKS after incubation with active calpain-2 in the presence of CaCl2 or with CaCl2 only for 1 h at 37°C. Lower-molecular weight bands above and below 50 kDa are shown in the lane with GST-MARCKS incubated with active calpain-2 in the presence of Ca2+ but absent in the lane with GST-MARCKS and Ca2+ alone. MWM, molecular weight markers. C: densitometric analysis of calpain proteolysis of MARCKS as shown in B. The data are representative of three independent experiments (n = 3). Data are represented as means ± SE. *P < 0.001 by t-test.

Fig. 2. SDS-PAGE and Coomassie staining analysis of ENaC cleavage by calpain-2. A: recombinant GST expressed and purified from BL21 cells are shown to have an electrophoretic mobility of 26 kDa. MWM, molecular weight markers. Recombinant GST-ENaCγ (B), GST-ENaCβ (C), and GST-ENaCα (D) fusion proteins purified from bacterial inclusion bodies were incubated with active calpain-2 in the presence of CaCl2 or with CaCl2 only for 1 h at 37°C. Lower-molecular weight bands corresponding to cleaved products from calpain-2 proteolysis are absent, and there is no appreciable difference between any of the three ENaC subunits incubated with or without calpain-2 in the presence of CaCl2. Active calpain-2 purified from porcine kidney was loaded separately as a control.
collected in freshly prepared ice-cold 1% Brij 96/TNE buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM sodium vanadate, and Halt protease and phosphatase inhibitor cocktail) at 4°C. The cellular lysate was passed 10 times through a 23-gauge syringe and then incubated on ice for 1 h. The lysate was centrifuged at 10,000 rpm at 4°C for 5 min to remove cellular debris, and 500 μL of the supernatant were mixed with an equal volume of freshly prepared 80% sucrose in TNE (without Brij 96 and without vanadate) before being transferred to a 13 × 23-mm Beckman centrifuge tube. Freshly prepared 35% sucrose in TNE (1,800 μL) was carefully applied to the top of the mixture followed by 500 μL of freshly prepared 5% sucrose. The sucrose gradient was centrifuged at 34,000 rpm at 4°C for 16 h in a SW50.1 rotor (Beckman). Fourteen fractions of 235-μL volumes were carefully collected from the top to the bottom of the tube. The first and last fractions were discarded, and the other fractions were analyzed by SDS-PAGE and Western blotting.

**MARCKS cleavage in native renal cells.** Mouse mpkCCDc14 cells were cultured on six-well plates to 70% confluency and transfected with a Flag-tagged MARCKS construct. The p3xFlag-CMV-10 plasmid was from Sigma-Aldrich. The original MARCKS construct was a gracious gift of Dr. Sumiko Watanabe, Tokyo University, Tokyo, Japan. We transfected cells with JetPrime reagent from Polyplus according to the manufacturer’s directions. When confluent, the cells were divided into three groups: untreated, treated with 100 μM hydrogen peroxide for 30 min, or treated with 100 μM hydrogen peroxide (30 min) plus 50 nM calpain inhibitor acetyl-calpastatin for 6 h. Cells were prepared for Western blotting and blotted with anti-Flag antibody (F-1804; Sigma-Aldrich) at a 1:1,000 dilution or anti-MARCKS antibody (ab-184546; Abcam) at a 1:1,000 dilution. The latter antibody was generated against a synthetic peptide within human MARCKS-like protein from amino acid 150 to the COOH terminus. The Universal Protein Resource (UniProt) database link is P49006.

**Statistical analysis.** All data are described as means ± SE (or SD where noted). Statistical analysis was performed using the Student’s t-test or, for multiple comparisons, Kruskal-Wallis one-way analysis of variance on ranks using SigmaStat software. Results were considered significant if P < 0.05.

**RESULTS**

**MARCKS is a substrate of calpain-2 in the presence of Ca2+**. Dulong et al. showed that human MARCKS is a substrate of μ-calpain in myoblasts (17). Consistent with this report, bioinformatics studies using a calpain-2 prediction algorithm suggested that MARCKS should be cleaved by calpain-2 in the kidney (data not shown). The ability of MARCKS to associate with the plasma membrane depends on its association with other proteins and posttranslational modifications, including phosphorylation and myristoylation (40). To determine whether MARCKS lacking myristoylation and phosphorylation is a substrate of calpain-2, we performed in vitro calpain-2 proteolytic assays using recombinant GST-MARCKS fusion protein and active calpain-2 protease. Because it is possible for calpain-2 to cleave itself, we incubated calpain-2 at 37°C in the presence of Ca2+ alone. In a parallel reaction, GST-MARCKS was incubated at 37°C with calpain-2 and Ca2+ for the same period of time. Lower-molecular weight bands above and below 50 kDa were observed in Coomassie blue-stained SDS-PAGE gels for GST-MARCKS incubated with calpain-2 in the presence Ca2+ (2.137 ± 0.209), but the proteolytic products were absent when MARCKS was incubated with Ca2+ alone (0.119 ± 0.00963, n = 3; Fig. 1). Here, we show that MARCKS is cleaved by calpain-2 in a calcium-dependent manner and its proteolysis does not require post-translational modifications.

**ENaC subunits are not substrates of Ca2+-dependent calpain-2.** To determine whether ENaC subunits are substrates of calpain-2 in the presence of Ca2+1, in vitro calpain-2 proteolytic assays were performed similar to those for recombinant MARCKS. Recombinant GST-ENaCα, GST-ENaCβ, and GST-ENaCγ fusion proteins were expressed and purified from bacterial inclusion bodies as previously described and incubated in the absence or presence of calpain-2 and Ca2+. Calpain-2 did not cleave any of the ENaC subunits in the

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**Fig. 3. Amiloride-sensitive transepithelial current measurements examining the effect of active calpain-2 or calpain-2 inhibition in mpkCCD cells.** A: active calpain-2 from porcine kidney was applied to the apical side of confluent mpkCCD cell monolayers. Transepithelial voltages and resistances were recorded, and equivalent transepithelial current was calculated using Ohm’s law. There was no change in current over time between cells treated with calpain-2 and untreated cells. Amiloride (0.5 μM) was applied to the apical side of the cells at the end of the experiment to show that current from any amiloride-insensitive nonselective cation channel was negligible. Current was calculated from three separate inserts (n = 3). B: effect of calpain-2 inhibition on amiloride-sensitive transepithelial current in mpkCCD cells by epithelial voltohmmeter measurements. Calpain-2 inhibitor (20 μM) or cytochalasin D (1.5 μM) as a control was applied to the apical side of confluent mpkCCD cell monolayers. Transepithelial current was calculated from transepithelial voltage and resistance measurements. At the end of the experiment, amiloride (0.5 μM) was added to show that the measured current was from amiloride-sensitive highly selective cation channels. Current was calculated from three separate inserts (n = 3) for each group. Data are represented as means ± SE. *P < 0.05 compared with untreated cells.
The presence of Ca\textsuperscript{2+} (Fig. 2). These studies show that unlike MARCKS, ENaC subunits are not cleaved by calcium-dependent calpain-2.

Calpain-2 exogenously applied to the surface of mpkCCD cells does not alter amiloride-sensitive transepithelial current. Calpain-2 activity is dependent on Ca\textsuperscript{2+}. The extracellular concentration of Ca\textsuperscript{2+} is a thousand-fold higher compared with the intracellular concentration. We examined whether or not calpain-2 could cleave the extracellular loops of ENaC subunits if it was present in the tubular lumen of the nephron. Calpain-2 purified from porcine kidney was applied to the apical side of mpkCCD cells, and transepithelial voltages and resistances across the confluent monolayers were measured after 15, 30, and 120 min. Amiloride (0.5 μM) was applied after the final time point, and transepithelial current was calculated using Ohm’s law. There was not a significant change in amiloride-sensitive transepithelial current in response to exogenous apical delivery of active calpain-2 protease compared with controls (n = 3 for each group; Fig. 3A). These results show that unlike various proteases that cleave the extracellular loops of ENaC and activate the channel, calpain-2 does not contribute to ENaC proteolysis and activation.

Calpain-2 inhibition decreases amiloride-sensitive transepithelial current in mpkCCD cells. To determine whether inhibiting endogenous calpain-2 activity has an effect on ENaC activity in mpkCCD cells, we treated cells with a pharmacological inhibitor of calpain-2, A6060, for different time intervals and measured transepithelial voltages and resistances across confluent cell monolayers. Transepithelial current was calculated using Ohm’s law while correcting for the diameter of the permeable Transwell support used to culture the cells. Amiloride (0.5 μM) was added to the cells at the end of the experiment to show that the measured current was from amiloride-sensitive ENaC. Compared with vehicle alone (DMSO; 8.2890 ± 0.0965), treatment with the calpain-2 inhibitor (6.3270 ± 0.2590) resulted in a decrease in transepithelial current 15 min after treatment (n = 3 for each group; Fig. 3B). This change was similar to the change observed when cells were treated with the actin cytoskeleton disruptor cytochalasin D, which was applied as a control (Fig. 3B). These studies show that pharmacological inhibition of calpain-2 decreases endogenous ENaC activity in a time-dependent manner.

Calpain-2 inhibition decreases ENaC activity in cell-attached patches recorded from Xenopus 2F3 cells. Xenopus 2F3 cells are a subclone of A6 cells that have been selected for high levels of amiloride-sensitive cation channels and represent an established model to examine endogenous ENaC activity. ENaC activity [measured as the product of the number of channels in a patch times the open probability (NP\textsubscript{o})] was significantly reduced in cells pretreated with the calpain-2 inhibitor compared with cells pretreated with vehicle alone (Fig. 4). NP\textsubscript{o}, was 0.52 ± 0.18 for the control group (n = 20) and 0.01 ± 0.01 for the calpain-2 inhibitor group (n = 13; Fig. 4B). These results show that calpain-2 is an important regulator of endogenous ENaC activity at the level of cell-attached single channels.

Calpain-2 alters the expression of MARCKS but not the expression of ENaC subunits. Goudenege et al. showed that MARCKS, m-calpain, and PKC\textalpha are present in caveolae fractions (22). To examine the effect of Ca\textsuperscript{2+}-dependent calpain-2 proteolysis on MARCKS and ENaC subunit protein expression, we performed sucrose density gradient ultracentrif-
Calpain-2 inhibition did not result in any changes in the protein expression of ENaC subunits (data not shown) but did alter the subcellular protein expression of MARCKS (Fig. 5). Calpain-2 inhibition reduced the expression of MARCKS in the first three light-density lipid-associated sucrose density gradient fractions (0.00037 ± 0.00004, 0.00127 ± 0.00027, and 0.00431 ± 0.00388) compared with vehicle (mock) treatment (0.03271 ± 0.00624, 0.03787 ± 0.00907, and 0.04188 ± 0.01049, respectively, n = 3 for each group; Fig. 5). Here, we show that pharmacological inhibition of calpain-2 prevents the association of MARCKS with lipids.

**Calpain-2 from porcine kidney does not directly bind phospholipid phosphates in vitro.** Calpain-2 was reported to associate with various phospholipids (7) and play a role in cleaving protein kinase C (27). To determine whether calpain-2 directly binds phospholipid phosphates and, if it binds to lipids, to which lipids, we performed PIP strip overlay binding assays (described in MATERIALS AND METHODS). Purified calpain-2 from porcine kidney was incubated with PIP strip nitrocellulose membranes in the presence of Ca²⁺. The blots were washed, incubated with calpain-2-specific antibody, followed by secondary antibody, and then developed to visualize immunoreactive spots corresponding to direct binding between the various lipids and calpain-2. Similar to GST alone, there was no appreciable binding between calpain-2 and the 15 different lipids tested (n = 3 strips for each group; Fig. 6, A and D). These results show that calpain-2 itself does not directly bind phospholipid phosphates.

**Ca²⁺-dependent calpain-2 cleavage of MARCKS promotes its interaction with specific lipids.** The myristoylation domain of MARCKS interacts hydrophobically with the cytosolic surface of the membrane phospholipid bilayer, whereas the effector domain interacts electrostatically with anionic lipids on the cytoplasmic face of the plasma membrane. MARCKS functions as a reversible source of PIP2 at the membrane, as each MARCKS molecule binds and sequesters three PIP2 molecules (31). PIP2 positively regulates ENaC gating (30, 37, 44), and MARCKS proteins allow for efficient interaction between PIP2 and ENaC (3, 4). To determine whether Ca²⁺-dependent calpain-2 proteolytic cleavage of MARCKS affects the ability of MARCKS to bind acidic phospholipids such as PIP2, we performed PIP strip overlay binding assays (as described in MATERIALS AND METHODS). As expected, and shown in Fig. 6, full-length GST-MARCKS bound strongly to 4,5-PIP2 [98 ± 4.8 units on a gray scale from 0 (white) to 256 (black), mean ± SD] and weakly or not at all to 3,4-PIP2 (22 ± 2.0 units, mean ± SD), 3,5-PIP2 (no binding detected), and 3,4,5-PIP3 (4.5 ± 0.89 units, mean ± SD). Calpain cleavage of MARCKS increases the binding to 4,5-PIP2 (134 ± 1.64 units, mean ± SD), 3,4-PIP2 (55 ± 5.6 units, mean ± SD), and 3,4,5-PIP3 (77 ± 7.9 units, mean ± SD), but not to 3,5-PIP2 (no binding detected). The binding between GST-MARCKS or calpain-cleaved GST-MARCKS and 3,4,5-PIP3 increased 2.5-fold, 4,5-PIP2 binding increased 1.36-fold, and 3,4,5-PIP3 binding increased 17-fold (n = 3 strips for each condition). These results show that proteolysis of MARCKS by calpain promotes MARCKS binding to most phosphatidylinositol lipid phosphates.

**Calpain-2 cleaves MARCKS and increases its association with ENaC.** The next aim was to determine whether or not calpain-2 serves as a negative feedback regulator in the PIP2-dependent regulation of ENaC mediated by MARCKS proteins. To corroborate the effect of calpain-2 proteolysis on the association between ENaC and MARCKS, we immunoprecipitated ENaC protein after pretreating Xenopus 2F3 cells with or without the pharmacological inhibitor of calpain-2, A6060, and then inducing Ca²⁺ mobilization with the Ca²⁺ ionophore A23187. The eluent from the immunoprecipitated complex was resolved by SDS-PAGE before probing for MARCKS by Western blotting. In Fig. 7 we immunoprecipitated ENaC and detected associated MARCKS in the immunoprecipitate when calpain-2 was activated or inhibited. We show that the association between ENaC and MARCKS decreased after pharmacological inhibition of calpain-2 [3.44 ± 0.0929; ratio of MARCKS to IgG band density; band density is mean value measured as pixel values on a scale from 0 (white) to 4,095 (black)] or after increasing intracellular Ca²⁺ mobilization (2.33 ± 0.172) compared with untreated cells (4.31 ± 0.110, n = 3) for each group. These data show that calpain-2 and intracellular calcium differentially regulate the association between ENaC and MARCKS.

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**Fig. 5.** Sucrose density gradient and Western blot analysis of calpain-2 inhibition on MARCKS protein expression in mpkCCD cells. A: representative Western blot (WB) showing MARCKS expression in various fractions including light-density sucrose gradient fractions. B: representative Western blot showing a substantial decrease in MARCKS protein expression in light-density sucrose gradient fractions after treating mpkCCD cells with the pharmacological inhibitor of calpain-2 A6060 compared with mock (vehicle) treatment. Flotillin was used as a marker of lipid rafts in A and B. C: densitometric analysis of the Western blot data (n = 3) presented in A. Data are represented as means ± SE. *P < 0.05, **P < 0.01.
Calpain-2 inhibition reduces MARCKS cleavage in renal cells. We examined MARCKS cleavage in mouse kidney cells transfected with a MARCKS construct with a COOH-terminal Flag tag. Our rationale was that if calpain-2 cleaved MARCKS between the effector domain and the COOH terminus, we should see two bands in blots in which we detect Flag: a full-length form and a small cleaved form that still has the Flag tag. If, in the same blots, we detect a MARCKS antibody whose epitope is near the NH2-terminal domain, we should see two bands: a full-length form and a much smaller cleavage product. The cells were divided into three groups: untreated, treated with 100 μM hydrogen peroxide for 30 min to activate calpain (42), or treated with 100 μM hydrogen peroxide (30 min) plus 50 nM calpain inhibitor acetyl-calpastatin for 6 h. We then blotted for Flag or MARCKS (n = 8; Fig. 8). To quantify the blots, we digitized the blots as 16-bit grayscale images and used ImageJ to measure the sum of all the pixel values that were above threshold in each band. We divided these values by 1,000. As we anticipated, with Flag we observed two bands, which we presume are uncleaved and partially cleaved MARCKS (6.50 ± 0.245 and 0.249 ± 0.0777, respectively, n = 3 for all measurements). After activation with H2O2, the uncleaved band density decreases slightly, and the partially cleaved product increases (6.13 ± 0.286 and 0.787 ± 0.218, respectively). In the presence of inhibitor, the uncleaved band density increases significantly, and the partially cleaved product is not detectable (7.52 ± 0.0639). Blotting for MARCKS, we observe two bands, one that corresponds to uncleaved MARCKS (band b graph; 4.60 ± 0.478 for untreated) and a lower-molecular-weight cleaved...
product (band c graph; 0.292 ± 0.0703 for untreated). After exposure to peroxide, uncleaved band b is unchanged (4.73 ± 0.335), and band c is slightly increased (0.541 ± 0.0391). In the presence of inhibitor, uncleaved band b is increased (5.99 ± 0.242), and cleaved band c is reduced (0.0323 ± 0.0067). These data confirm that in renal epithelial cells, MARCKS is cleaved by calpains between the effector domain and the COOH terminus of the protein.

**Calpain-2 inhibition decreases MARCKS cleavage in the kidney of wild-type mice.** Our results so far have demonstrated a role for calpain cleavage of MARCKS in regulating ENaC in a tissue culture model of sodium transport, but the role of calpain-2 in regulating MARCKS expression in the kidney has not been characterized. Therefore we examined MARCKS protein expression in the kidney of SV129 wild-type mice. We used osmotic minipumps to deliver either PBS or calpain inhibitor in SV129 wild-type mice. The mice were euthanized, and the kidneys were harvested for protein. MARCKS protein expression was examined by Western blotting. Compared with SV129 mice that received infusion of vehicle alone (6.623 ± 0.655), infusion of the calpain-2 inhibitor A6060 in SV129 mice decreased the density of a lower-molecular-weight immunoreactive band at 37 kDa in blots probed with MARCKS antibody (3.237 ± 0.449, n = 3 for each group; Fig. 9). These data support the cleavage of MARCKS by calpains in the in vivo kidney of wild-type mice.

**DISCUSSION**

Phosphatidylinositol phosphates (PIPs) have been shown to activate or inhibit several different ion channels and transporters (24). The epithelial sodium channel is among one of these transport mechanisms activated by PIPs (30, 37, 44). PIP2 binds to the intracellular amino and carboxy termini of ENaC subunits at the membrane to keep the channel in an open conformation. The interaction between ENaC and PIP2 is regulated by the MARCKS family of proteins; these adaptor proteins increase the local concentration of PIP2 at the membrane allowing for a more efficient interaction. MARCKS and MLP sequestering of PIP2 may prevent its hydrolysis by phospholipase C and decreases inositol trisphosphate and diacylglycerol production. The ability of MARCKS and MLP1 to function as adaptor proteins at the membrane depends on posttranslational modifications and interactions with other proteins. In the former mechanism, myristoylation at the amino terminus promotes hydrophobic interactions with the membrane while phosphorylation at serine residues within the effector domain of the protein by PKC prevents electrostatic interaction with the membrane. In the latter mechanism, direct binding between Ca²⁺-calmodulin and specific amino acid residues within the effector domain of MARCKS causes displacement of the protein from the membrane and translocation to the cytoplasm.

Calpains perform a more specialized role than intracellular enzymes that degrade other proteins and render them inactive. Instead, calpains are able to orchestrate signal transduction cascades in response to changes in intracellular Ca²⁺ concentration. Our data are consistent with those of other groups as we show here that MARCKS is a substrate of calpain-2 (Fig. 1). The regulation of sodium transport mechanisms in the kidney by calpain-2-dependent proteolysis of MARCKS has
MARCKS band b
minipump infusion

+ when mpkCCD cells were treated with vehicle alone, Fig. 5A with the amino acids involved in lipid binding. As shown in would suggest that the calpain-2 cleavage sites do not overlap domain from directly binding anionic phospholipids. This pain-2 cleavage of MARCKS does not prevent its effector not been investigated before this study. ENaC plays an important role in total body sodium homeostasis and blood pressure control. The physiological significance of ENaC is underscored by its dysfunction in inherited forms of blood pressure disorders including Liddle’s syndrome and pseudohypoaldosteronism. Our data presented here show for the first time that calpain-2 modulates the direct binding between MARCKS and anionic lipids (Fig. 6). Surprisingly, the Ca\(^{2+}\)-dependent calpain-2 cleavage of MARCKS does not prevent its effector domain from directly binding anionic phospholipids. This would suggest that the calpain-2 cleavage sites do not overlap with the amino acids involved in lipid binding. As shown in Fig. 5A, when mpkCCD cells were treated with vehicle alone, MARCKS, presumably cleaved by Ca\(^{2+}\)-dependent calpain-2, was expressed in light-density sucrose gradient fractions containing lipids and lipid-associated proteins. This is one of multiple Ca\(^{2+}\)-sensitive mechanisms that can regulate the PIP2-dependent gating of ENaC by the MARCKS family of proteins. A second Ca\(^{2+}\)-dependent mechanism for the regulation of MARCKS involves the translocation of Ca\(^{2+}\)-calmodulin to the membrane, where it binds the effector domain of MARCKS causing the protein to lose its affinity with the membrane (31). Finally, we demonstrated that a third Ca\(^{2+}\)- dependent regulatory mechanism for MARCKS exists in the kidney. We recently showed a role for CaMKII in the regulation of MARCKS/MLP1 and ENaC (4). CaMKII activation is

![Image of Figure 8](http://ajpcell.physiology.org/)

**Fig. 8.** Calpain-mediated MARCKS cleavage in native renal cells. Mouse mpkCCDc14 cells were transfected with a Flag-tagged MARCKS construct. Confluent cells were divided into three groups: untreated (black bars), treated with 100 μM hydrogen peroxide for 30 min to activate calpain, or treated with 100 μM hydrogen peroxide (30 min) plus 50 nM calpain inhibitor acetyl-calpastatin for 2–12 h. We then blotted for Flag or MARCKS. With Flag we observed two bands, which we presume are uncleaved and partially cleaved MARCKS (band a, uncleaved; band #, cleaved). In the presence of inhibitor the uncleaved MARCKS increases significantly, and the partially cleaved MARCKS decreases [data from 6 blots (n = 6) determined by Kruskal-Wallis ANOVA on ranks with Student-Neuman-Keuls posttest]. After stripping the Flag blots, we blotted for MARCKS and observed two bands: one that corresponds to uncleaved MARCKS (band b) and a lower-molecular-weight cleaved product (band c) that increases after exposure to peroxide and is reduced in the presence of inhibitor [data from 6 blots determined by Kruskal-Wallis ANOVA on ranks with Student-Neuman-Keuls posttest). Units are equivalent for all bars in graphs. Data are represented as means ± SE. *P < 0.05.

![Image of Figure 9](http://ajpcell.physiology.org/)

**Fig. 9.** Western blot analysis of MARCKS protein expression in native kidney after infusion of the calpain-2 inhibitor A6060 in mice. Osmotic minipumps were used to infuse the calpain-2 inhibitor A6060 or 50% DMSO in sterile saline in SV129 mice at a rate of 0.5 μl/h for 7 days. A: representative Western blot of three independent experiments probing for MARCKS protein expression shows immunoreactive bands at 75 (doublet), 60, and 37 kDa. The density of the 37-kDa cleaved form of MARCKS was less in lysates from kidneys infused with calpain-2 inhibitor compared with PBS in SV129 mice. B: densitometric analysis of the immunoreactive band at 37 kDa in A representative of three independent experiments (n = 3). Data are represented as means ± SE. *P < 0.05 determined by t-test.

**WB: MARCKS**

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dependent on Ca\(^{2+}\) and calmodulin, and we showed that the phosphorylation of filamin by CaMKII causes a rearrangement of the cytoskeleton and disrupts the association between ENaC and MARCKS at the membrane (4). It is not surprising that multiple Ca\(^{2+}\)-dependent mechanisms exist to regulate sodium transport across the membrane of polarized epithelial cells of the kidney. This shows that the cell does not depend on a single mechanism for such a physiologically important process but, instead, multiple mechanisms exist to possibly compensate for one another if necessary. We previously showed that PKC activity reduces the association between ENaC and MARCKS, presumably from MARCKS translocating to the cytoplasm after serine residues within the effector domain are phosphorylated (3). PKC was shown to be a substrate of calpain (27, 33, 39). The degradation of PKC\(\alpha\) by calpain would increase the amount of MARCKS at the membrane provided that other Ca\(^{2+}\)-dependent mechanisms such as Ca\(^{2+}\)-calmodulin binding to MARCKS or activation of CaMKII do not compensate for the loss of PKC\(\alpha\) activity.

Arguments can be made either in support of autoproteolysis being a mechanism for activation of calpains or against autoproteolysis playing a role in enzyme activation (11). We did not observe autoproteolysis of calpain-2 after incubating the active protease with Ca\(^{2+}\) (Fig. 1). We did, however, observe lower-molecular-weight bands in our SDS-PAGE analysis after incubating recombinant GST-MARCKS fusion proteins with active calpain-2 in the presence of Ca\(^{2+}\) (Fig. 1). This may suggest that Ca\(^{2+}\) may be required as a cofactor for proteolytic activity, but not for autoproteolysis of the calpain-2.

The inhibition of calpain was shown to reduce MARCKS phosphorylation in rat brain tissue (16). This would cause MARCKS to accumulate at the membrane. Conversely, we show that calpain-2 inhibition decreases MARCKS association with ENaC as does intracellular calcium mobilization (Fig. 7). There are multiple explanations for these different observations. First, the different isoforms of calpain and MARCKS are tissue specific and may also be species specific. In Xenopus 2F3 cells we show expression of MARCKS protein and at least

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**Fig. 10. Proposed model describing the role of calpain-2 in the MARCKS-mediated PIP2-dependent regulation of ENaC in the kidney.**

**A:** in the presence of active calpain-2 a region downstream of the effector domain of MARCKS is cleaved. Calpain-2 proteolysis of MARCKS prevents PKC\(\alpha\) from accessing and phosphorylating serine residues within the effector domain resulting in constitutive or increased MARCKS association with the membrane. At the apical plasma membrane, MARCKS sequesters acidic phospholipid phosphates (e.g., PIP2) and presents them to ENaC. These phospholipid phosphates positively regulate the gating of ENaC and maintain the channel in an open conformation. **B:** conversely, in the absence of active calpain-2, MARCKS is not cleaved, and the carboxy-terminal tail potentiates the access of PKC\(\alpha\) to the effector domain of MARCKS. PKC\(\alpha\) phosphorylates serine residues within the effector domain of MARCKS and causes the protein to lose affinity for the membrane and translocate to the cytoplasm. In turn, the interaction between PIP2 and ENaC is reduced because both molecules are rare and do not associate by random diffusion alone. PIP2 is shown to be randomly dispersed within the inner leaflet of the apical plasma membrane instead of being in close proximity to ENaC after MARCKS is phosphorylated by PKC and translocated to the cytoplasm. C-terminus, COOH.
one other member of the same family known as MLP1 (3). In mouse mpkCCD cells, MLP1 is predominantly expressed. Second, there is tissue specificity of the different protein kinase C isoforms, which plays an important role in modulating the translocation of MARCKS from the membrane. In the kidney, PKCα is the main isoform expressed, whereas other isoforms of PKC are differentially expressed in other tissues. Dulongs et al. showed that calpain cleavage favors cytoplasmic MARCKS phosphorylated at serine residues by PKC (17). This implies that any interaction that stabilizes MARCKS at the membrane would affect its cleavage by calpain. We and others have shown that MARCKS associates with cytoskeleton-associated proteins and lipids at the plasma membrane. The actin cytoskeleton and lipid composition may vary considerably between tissue types as would the amount of MARCKS at the membrane and cytoplasm.

A role for calpain 2 in regulating the cytoskeleton in mouse lung epithelial cells has been described (18). Interestingly, several cytoskeleton-associated proteins are substrates of calpains including fodrin (2), MARCKS (17, 29), and filamin (21). Several studies have shown that ENaC is regulated by the actin cytoskeleton (13, 26, 38). The ability of calpains to cleave cytoskeleton-associated proteins may allow for the reorganization of actin cytoskeleton. This may affect the expression and function of membrane proteins including transporters and ion channels by multiple mechanisms. First, the reorganization of the cytoskeleton may disrupt protein-protein interactions that are important for stabilizing a complex of proteins at the membrane. Second, the reorganization of the cytoskeleton may trigger conformational changes in the protein itself to either promote or attenuate the function of the protein. Interestingly, many of the cytoskeleton-associated proteins known to play a role in the reorganization of the actin cytoskeleton including MARCKS, fodrin, and filamin are also regulated by phosphorylation (3, 4, 38). A putative role for calpains in regulating kinase activity and subsequently phosphorylation of cytoskeleton-associated proteins warrants further examination.

In native cells, MARCKS is cleaved by calpain-2 to a higher-molecular-weight form that still contains the Flag epitope and a lower-molecular-weight form that cannot be detected by Flag antibody but can still be detected by a MARCKS antibody (Fig. 8). This is not surprising because the Flag tag is on the NH₂-terminal end of MARCKS and the MARCKS antibody recognizes an epitope in the COOH-terminal end. Reactive oxygen species such as hydrogen peroxide activate calpains. The addition of hydrogen peroxide does not dramatically change calpain-2-mediated MARCKS cleavage and this implies that calpain-2 must be fairly active under basal conditions. In fact, calpain-2 inhibition can increase uncleaved MARCKS and reduce cleaved MARCKS (Fig. 8).

Here, we examined the regulation of ENaC activity by calpain-2-mediated proteolysis of MARCKS in cultured renal cells. In the presence of active calpain-2, MARCKS is cleaved at specific sites downstream of its effector domain. This presumably reduces the ability of PKC to access the effector domain and phosphorylate serine residues, which would lead to the translocation of MARCKS from the plasma membrane to the cytoplasm (Fig. 10). Indeed, we show that under basal conditions where calpain-2 is active, MARCKS associates with lipid fractions of cultured renal epithelial cells but this association is attenuated when calpain-2 is inhibited (Fig. 5). The presence of MARCKS at the plasma membrane is essential for concentrating PIP2 and preventing its hydrolysis by phospholipase C. MARCKS promotes the interaction between PIP2 and ENaC, which positively regulates gating of the channel. Other studies presented here suggest that calpain-2 cleaves MARCKS in the mouse kidney, but additional in vivo and ex vivo studies are necessary to further examine ENaC activity in the aldosterone-sensitive distal nephron.

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Calpain-2 proteolysis of MARCKS regulates ENaC


